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METHODS AND COMPOSITIONS FOR THE  
PRODUCTION OF STABLY TRANSFORMED,  
FERTILE MONOCOT PLANTS AND CELLS THEREOF

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## BACKGROUND OF THE INVENTION

The present application is a continuation-in-part of U.S. Serial Number 07/565,844, filed August 9, 1990, which was a continuation-in-part of U.S. Serial Number 07/513,298, filed April 17, 1990, ~~which was a continuation-in-part of U.S. Serial Number 07/392,176, filed August 9, 1989.~~

### 1. Field of the Invention

The present invention relates to reproducible systems for genetically transforming monocotyledonous plants such as maize, to methods of selecting stable genetic transformants from suspensions of transformed cells, and to methods of producing fertile plants from the transformed cells. Exemplary transformation methods include the use of microprojectile bombardment to introduce nucleic acids into cells, and selectable and/or screenable marker systems, for example, genes which confer resistance (e.g., antibiotic, herbicide, etc.), or which contain an otherwise phenotypically observable or other detectable trait. In other aspects, the invention relates to the production of stably transformed and fertile monocot plants, gametes and offspring from the transgenic plants.

### 2. Description of the Related Art

Ever since the human species emerged from the hunting-gathering phase of its existence, and entered an agricultural phase, a major goal of human ingenuity and invention has been to improve crop yield and to alter and improve the characteristics of plants. In particular, man has sought to alter the characteristics of plants to make them more tasty and/or nutritious, to produce increased crop yield or render plants more adaptable to specific environments.

Up until recent times, crop and plant improvements depended on selective

breeding of plants with desirable characteristics. Initial breeding success was probably accidental, resulting from observation of a plant with desirable characteristics, and use of that plant to propagate the next generation. However, because such plants had within them heterogenous genetic complements, it was unlikely that progeny identical to the parent(s) with the desirable traits would emerge. Nonetheless, advances in controlled breeding have resulted from both increasing knowledge of the mechanisms operative in hereditary transmission, and by empirical observations of results of making various parental plant crosses.

Recent advances in molecular biology have dramatically expanded man's ability to manipulate the germplasm of animals and plants. Genes controlling specific phenotypes, for example specific polypeptides that lend antibiotic or herbicide resistance, have been located within certain germplasm and isolated from it. Even more important has been the ability to take the genes which have been isolated from one organism and to introduce them into another organism. This transformation may be accomplished even where the recipient organism is from a different phylum, genus or species from that which donated the gene (heterologous transformation).

Attempts have been made to genetically engineer desired traits into plant genomes by introduction of exogenous genes using genetic engineering techniques. These techniques have been successfully applied in some plant systems, principally in dicotyledonous species. The uptake of new DNA by recipient plant cells has been accomplished by various means, including *Agrobacterium* infection (Nester *et al.*, 1984), polyethylene glycol (PEG)-mediated DNA uptake (Lorz *et al.*, 1985), electroporation of protoplasts (Fromm *et al.*, 1986) and microprojectile bombardment (Klein *et al.*, 1987). Unfortunately, the introduction of exogenous DNA into monocotyledonous species and subsequent regeneration of transformed plants has proven much more difficult than transformation and regeneration in dicotyledonous plants. Moreover, reports of methods for the transformation of



monocotyledons such as maize, and subsequent production of fertile maize plants, have not been forthcoming. Consequently, success has not been achieved in this area and commercial implementation of transformation by production of fertile transgenic plants has not been achieved. This failure has been particularly  
5 unfortunate in the case of maize, where there is a particularly great need for methods for improving genetic characteristics.

Problems in the development of genetically transformed monocotyledonous species have arisen in a variety of general areas. For example, there is generally a  
10 lack of methods which allow one to introduce nucleic acids into cells and yet permit efficient cell culture and eventual regeneration of fertile plants. Only limited successes have been noted. In rice, for example, DNA transfer has only recently been reported using protoplast electroporation and subsequent regeneration of transgenic plants (Shimamoto *et al.*, 1989). Furthermore, in maize, transformation  
15 using protoplast electroporation has also been reported (see, e.g., Fromm *et al.*, 1986).

However, recovery of stably transformed plants has not been reproducible. A particularly serious failure is that the few transgenic plants produced in the case  
20 of maize have not been fertile (Rhodes *et al.*, 1988). While regeneration of fertile corn plants from protoplasts has been reported (Prioli & Sondahl, 1989; Shillito *et al.*, 1989), these reported methods have been limited to the use of non-transformed protoplasts. Moreover, regeneration of plants from protoplasts is a technique which carries its own set of significant drawbacks. Even with vigorous  
25 attempts to achieve fertile, transformed maize plants, reports of success in this regard have not been forthcoming.

A transformation technique that circumvents the need to use protoplasts is microprojectile bombardment. Although transient expression of a reporter gene  
30 was detected in bombarded tobacco pollen (Twell *et al.*, 1989), stable

transformation by microprojectile bombardment of pollen has not been reported for any plant species. Bombardment of soybean apical meristems with DNA-coated gold particles resulted in chimeric plants containing transgenic sectors. Progeny containing the introduced gene were obtained at a low frequency (McCabe *et al.*, 1988). Bombardment of shoot meristems of immature maize embryos resulted in sectors of tissue expressing a visible marker, anthocyanin, the synthesis of which was triggered by the introduction of a regulatory gene (Tomes, 1990). An analysis of cell lineage patterns in maize (McDaniel & Poethig, 1988) suggests that germline transformation of maize by such an approach may be difficult.

A second major problem in achieving successful monocot transformation has resulted from the lack of efficient marker gene systems which have been employed to identify stably transformed cells. Marker gene systems are those which allow the selection of, and/or screening for, expression products of DNA. For use as assays for transformed cells, the selectable or screenable products should be those from genetic constructs introduced into the recipient cells. Hence, such marker genes can be used to identify stable transformants.

Of the more commonly used marker gene systems are gene systems which confer resistance to aminoglycosides such as kanamycin. While kanamycin resistance has been used successfully in both rice (Yang *et al.*, 1988) and corn protoplast systems (Rhodes *et al.*, 1988), it remains a very difficult selective agent to use in monocots due to high endogenous resistance (Hauptmann, *et al.*, 1988). Many monocot species, maize, in particular, possess high endogenous levels of resistance to aminoglycosides. Consequently, this class of compounds cannot be used reproducibly to distinguish transformed from non-transformed tissue. New methods for reproducible selection of or screening for transformed plant cells are therefore needed.

Accordingly, it is clear that improved methods and/or approaches to the genetic transformation of monocotyledonous species would represent a great advance in the art. Furthermore, it would be of particular significance to provide novel approaches to monocot transformation, such as transformation of maize cells, which would allow for the production of stably transformed, fertile corn plants and progeny into which desired exogenous genes have been introduced. Furthermore, the identification of marker gene systems applicable to monocot systems such as maize would provide a useful means for applying such techniques generally. Thus, the development of these and other techniques for the preparation of stable genetically transformed monocots such as maize could potentially revolutionize approaches to monocot breeding.

## SUMMARY OF THE INVENTION

The present invention addresses one or more of the foregoing or other shortcomings in the prior art by providing compositions and methods for the preparation of stably transformed, monocotyledonous cells and the subsequent regeneration of fertile, transgenic plants and progeny, particularly maize. The invention particularly provides techniques for the preparation of transgenic, fertile monocots, such as maize, which have been stably transformed through the introduction of discrete DNA sequences into the plant genome.

The invention thus relates generally to methods for the production of transgenic plants. As used herein, the term "transgenic plants" is intended to refer to plants that have incorporated DNA sequences, including but not limited to genes which are perhaps not normally present, DNA sequences not normally transcribed into RNA or translated into a protein ("expressed"), or any other genes or DNA sequences which one desires to introduce into the non-transformed plant, such as genes which may normally be present in the non-transformed plant but which one desires to either genetically engineer or to have altered expression. It is contemplated that in some instances the genome of transgenic plants of the present invention will have been augmented through the stable introduction of the transgene. However, in other instances, the introduced gene will replace an endogenous sequence.

Exemplary genes which may be introduced include, for example, DNA sequences or genes from another species, or even genes or sequences which originate with or are present in the same species, but are incorporated into recipient cells by genetic engineering methods rather than classical reproduction or breeding techniques. However, the term "exogenous", is also intended to refer to genes which are not normally present in the cell being transformed, or perhaps simply not present in the form, structure, etc., as found in the transforming DNA

segment or gene, or genes which are normally present yet which one desires, e.g., to have overexpressed. Thus, the term "exogenous" gene or DNA refers to any gene or DNA segment that is introduced into a recipient cell, regardless of whether a similar gene may already be present in such a cell. Introduced, in this context, is known in the art to mean introduced by the hand of man.

The most preferred monocots for use in the present invention will be cereals such as maize. The present invention is exemplified through the use of A188 x B73 cell lines, cell lines developed from other genotypes and immature embryos. Hence, it will be understood that the invention is in no way limited to a particular genotype or cell line. To date, a variety of different *Zea mays* lines and germplasms have been tested for their ability to be successfully employed in the preparation of fertile, transgenic corn. The status of these studies is set forth in some detail hereinbelow. Generally speaking, these studies have demonstrated that 24 out of 36 maize cultures were transformable. Of those cell lines tested, 11 out of 20 have produced fertile plants.

One exemplary embodiment for generating a stably transformed monocot includes culturing recipient corn cells in suspension cultures using embryogenic cells in Type II callus, selecting for small (10-30 $\mu$ ) isodiametric, cytoplasmically dense cells, introducing a desired DNA segment into these cells, growing the transformed cells in or on culture medium containing hormones, subculturing into a progression of media to facilitate development of shoots and roots, and finally, hardening the transgenic plant and readying it metabolically for growth in soil.

The present invention is suitable for use in transforming any maize variety. While not all cell lines developed out of a particular variety or cross will necessarily show the same degree of stable transformability, it has been the inventors' finding that a reasonable percentage of cell lines developed from essentially every genotype tested to date can be developed into fertile, transgenic plants. Thus,

where one desires to prepare transformants in a particular cross or variety, it will generally be desirable to develop several cell lines from the particular cross or variety (e.g., 8 to 10), and subject all of the lines so developed to the transformation protocols hereof.

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Another exemplary embodiment for generating a stably transformed monocot includes introducing a desired DNA segment into cells of organized tissues such as immature embryos, growing the embryos on a culture medium, subculturing into a progression of media to facilitate development of shoots and roots, and finally,  
10 hardening the transgenic plant and readying it metabolically for growth in soil. In this embodiment the invention is capable of transforming any variety of maize. Through the use of the present invention it is possible to simultaneously deliver DNA segments to a large number of embryos. It has been the inventor's finding that a percentage of the embryos that are contacted by exogenous DNA will  
15 develop into fertile transgenic plants, similar to delivering DNA to a large population of cultured cells. The present invention is exemplified through the use of immature embryos from the genotypes H99 and Hi-II, but is in no way limited to these genotypes. To date only experiments with these genotypes have progressed to the point where one would reasonably expect to recover transformants.  
20 Generally speaking one would expect to be able to recover fertile transgenic plants from any variety of maize.

Moreover, the ability to provide even a single fertile, transgenic corn line would be generally sufficient to allow the introduction of the transgenic component  
25 (e.g., recombinant DNA) of that line into a second corn line of choice. This is because by providing fertile, transgenic offspring, the practice of the invention allows one to subsequently, through a series of breeding manipulations, move a selected gene from one corn line into an entirely different corn line. For example, studies have been conducted wherein the gene for resistance to the herbicide  
30 Basta®, *bar*, has been moved from two transformants derived from cell line SC716

and one transformant derived from cell line SC82 into 18 elite inbred lines by backcrossing. It is possible with these inbreds to produce a large number of hybrids. Eleven such hybrids have been made and are in field tests.

## 5     **I.     Recipient Cells**

Practicing the present invention includes the generation and use of recipient cells. As used herein, the term "recipient cells" refers to monocot cells that are receptive to transformation and subsequent regeneration into stably transformed,  
10     fertile monocot plants.

### **A.     Sources of Cells**

Recipient cell targets include, but are not limited to, meristem cells, Type I,  
15     Type II, and Type III callus, immature embryos and gametic cells such as microspores pollen, sperm and egg cells. Type I, Type II, and Type III callus may be initiated from tissue sources including, but not limited to, immature embryos, seedling apical meristems, microspores and the such. Those cells which are capable of proliferating as callus are also recipient cells for genetic transformation.  
20     The present invention provides techniques for transforming immature embryos followed by initiation of callus and subsequent regeneration of fertile transgenic plants. Direct transformation of immature embryos obviates the need for long term development of recipient cell cultures. Pollen, as well as its precursor cells, microspores, may be capable of functioning as recipient cells for genetic  
25     transformation, or as vectors to carry foreign DNA for incorporation during fertilization. Direct pollen transformation would obviate the need for cell culture. Meristematic cells (i.e., plant cells capable of continual cell division and characterized by an undifferentiated cytological appearance, normally found at growing points or tissues in plants such as root tips, stem apices, lateral buds,  
30     etc.) may represent another type of recipient plant cell. Because of their

undifferentiated growth and capacity for organ differentiation and totipotency, a single transformed meristematic cell could be recovered as a whole transformed plant. In fact, it is proposed that embryogenic suspension cultures may be an *in vitro* meristematic cell system, retaining an ability for continued cell division in an undifferentiated state, controlled by the media environment.

In certain embodiments, cultured plant cells that can serve as recipient cells for transforming with desired DNA segments include corn cells, and more specifically, cells from Zea mays L. Somatic cells are of various types.

Embryogenic cells are one example of somatic cells which may be induced to regenerate a plant through embryo formation. Non-embryogenic cells are those which will typically not respond in such a fashion. An example of non-embryogenic cells are certain Black Mexican Sweet (BMS) corn cells. These cells have been transformed by microprojectile bombardment using the *neo* gene followed by selection with the aminoglycoside, kanamycin (Klein *et al.*, 1989). However, this BMS culture was not found to be regenerable.

The development of embryogenic maize calli and suspension cultures useful in the context of the present invention, e.g., as recipient cells for transformation, has been described in U.S. ~~Serial No. 06/877,033, filed 06/07/86~~, <sup>Patent no. 5,134,074</sup> incorporated herein by reference.

The present invention also provides certain techniques that may enrich recipient cells within a cell population. For example, Type II callus development, followed by manual selection and culture of friable, embryogenic tissue, generally results in an enrichment of recipient cells for use in, e.g., micro-projectile transformation. Suspension culturing, particularly using the media disclosed herein, may also improve the ratio of recipient to non-recipient cells in any given population. Manual selection techniques which employed to select recipient cells may include, e.g., assessing cell morphology and differentiation, or may use



various physical or biological means. Cryopreservation is also contemplated as a possible method of selecting for recipient cells.

Manual selection of recipient cells, e.g., by selecting embryogenic cells from the surface of a Type II callus, is one means employed by the inventors in an attempt to enrich for recipient cells prior to culturing (whether cultured on solid media or in suspension). The preferred cells may be those located at the surface of a cell cluster, and may further be identifiable by their lack of differentiation, their size and dense cytoplasm. The preferred cells will generally be those cells which are less differentiated, or not yet committed to differentiation. Thus, one may wish to identify and select those cells which are cytoplasmically dense, relatively unvacuolated with a high nucleus to cytoplasm ratio (e.g., determined by cytological observations), small in size (e.g., 10-20  $\mu\text{m}$ ), and capable of sustained divisions and somatic proembryo formation.

It is proposed that other means for identifying such cells may also be employed. For example, through the use of dyes, such as Evan's blue, which are excluded by cells with relatively non-permeable membranes, such as embryogenic cells, and taken up by relatively differentiated cells such as root-like cells and snake cells (so-called due to their snake-like appearance).

Other possible means of identifying recipient cells include the use of isozyme markers of embryogenic cells, such as glutamate dehydrogenase, which can be detected by cytochemical stains (Fransz *et al.*, 1989). However, it is cautioned that the use of isozyme markers such as glutamate dehydrogenase may lead to some degree of false positives from non-embryogenic cells such as rooty cells which nonetheless have a relatively high metabolic activity.

## B. Media

In certain embodiments, recipient cells are selected following growth in culture. Where employed, cultured cells will preferably be grown either on solid supports or in the form of liquid suspensions. In either instance, nutrients may be provided to the cells in the form of media, and environmental conditions controlled. There are many types of tissue culture media comprised of amino acids, salts, sugars, growth regulators and vitamins. Most of the media employed in the practice of the invention will have some similar components (see, e.g., Table 1 herein below), the media differ in the composition and proportions of their ingredients depending on the particular application envisioned. For example, various cell types usually grow in more than one type of media, but will exhibit different growth rates and different morphologies, depending on the growth media. In some media, cells survive but do not divide.

Various types of media suitable for culture of plant cells have been previously described. Examples of these media include, but are not limited to, the N6 medium described by Chu *et al.* (1975) and MS media (Murashige & Skoog, 1962). The inventors have discovered that media such as MS which have a high ammonia/nitrate ratio are counterproductive to the generation of recipient cells in that they promote loss of morphogenic capacity. N6 media, on the other hand, has a somewhat lower ammonia/nitrate ratio, and is contemplated to promote the generation of recipient cells by maintaining cells in a proembryonic state capable of sustained divisions.

## C. Cell Cultures

### 1. Initiation

5 In the practice of the invention it is sometimes, but not always, necessary to develop cultures which contain recipient cells. Suitable cultures can be initiated from a number of whole plant tissue explants including, but not limited to, immature embryos, leaf bases, immature tassels, anthers, microspores, and other tissues containing cells capable of *in vitro* proliferation and regeneration of fertile  
10 plants. In one exemplary embodiment, recipient cell cultures are initiated from immature embryos of *Zea mays* L. by growing excised immature embryos on a solid culture medium containing growth regulators including, but not limited to, dicamba, 2,4-D, NAA, and IAA. In some instances it will be preferred to add silver nitrate to culture medium for callus initiation as this compound has been  
15 reported to enhance culture initiation (Vain et al., 1989). Embryos will produce callus that varies greatly in morphology including from highly unorganized cultures containing very early embryogenic structures (such as, but not limited to, type II cultures in maize), to highly organized cultures containing large late embryogenic structures (such as, but not limited to, type I cultures in maize). This variation in  
20 culture morphology may be related to genotype, culture medium composition, size of the initial embryos and other factors. Each of these types of culture morphologies is a source of recipient cells.

The development of suspension cultures capable of plant regeneration may  
25 be used in the context of the present invention. Suspension cultures may be initiated by transferring callus tissue to liquid culture medium containing growth regulators. Addition of coconut water or other substances to suspension culture medium may enhance growth and culture morphology, but the utility of suspension cultures is not limited to those containing these compounds. In some  
30 embodiments of this invention, the use of suspension cultures will be preferred as

these cultures grow more rapidly and are more easily manipulated than callus cells growing on solid culture medium.

When immature embryos or other tissues directly removed from a whole plant are used as the target tissue for DNA delivery, it will only be necessary to initiate cultures of cells insofar as is necessary for identification and isolation of transformants. In an illustrative embodiment, DNA is introduced by particle bombardment into immature embryos following their excision from the plant. Embryos are transferred to a culture medium that will support proliferation of tissues and allow for selection of transformed sectors, 0-14 days following DNA delivery. In this embodiment of the invention it is not necessary to establish stable callus cultures capable of long term maintenance and plant regeneration.

## **2. Maintenance**

The method of maintenance of cell cultures may contribute to their utility as sources of recipient cells for transformation. Manual selection of cells for transfer to fresh culture medium, frequency of transfer to fresh culture medium, composition of culture medium, and environment factors including, but not limited to, light quality and quantity and temperature are all important factors in maintaining callus and/or suspension cultures that are useful as sources of recipient cells. It is contemplated that alternating callus between different culture conditions may be beneficial in enriching for recipient cells within a culture. For example, it is proposed that cells may be cultured in suspension culture, but transferred to solid medium at regular intervals. After a period of growth on solid medium cells can be manually selected for return to liquid culture medium. It is proposed that by repeating this sequence of transfers to fresh culture medium it is possible to enrich for recipient cells. It is also contemplated that passing cell cultures through a 1.9 mm sieve is useful in maintaining the friability of a callus or

suspension culture and may be beneficial is enriching for transformable cells.

### 3. Cryopreservation

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Additionally, the inventors propose that cryopreservation may effect the development of, or perhaps select for, recipient cells. Cryopreservation selection may operate due to a selection against highly vacuolated, non-embryogenic cells, which may be selectively killed during cryopreservation. The inventors propose that there is a temporal window in which cultured cells retain their regenerative ability, thus, it is believed that they must be preserved at or before that temporal period if they are to be used for future transformation and regeneration.

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For use in transformation, suspension or callus culture cells may be cryopreserved and stored for periods of time, thawed, then used as recipient cells for transformation. An illustrative embodiment of cryopreservation methods comprises the steps of slowly adding cryoprotectants to suspension cultures to give a final concentration of 10% dimethyl sulfoxide, 10% polyethylene glycol (6000MW), 0.23 M proline and 0.23 M glucose. The mixture is then cooled to -35°C at 0.5°C per minute. After an isothermal period of 45 minutes, samples are placed in liquid N<sub>2</sub> (modification of methods of Withers and King (1979); and Finkle *et al.*(1985)). To reinitiate suspension cultures from cryopreserved material, cells may be thawed rapidly and pipetted onto feeder plates similar to those described by Rhodes *et al.* (Vaeck *et al.*, 1987).

## II. DNA Sequences

Virtually any DNA composition may be used for delivery to recipient monocotyledonous cells to ultimately produce fertile transgenic plants in accordance with the present invention. For example, DNA segments in the form of vectors and plasmids, or linear DNA fragments, in some instances containing only the DNA element to be expressed in the plant, and the like, may be employed.

In certain embodiments, it is contemplated that one may wish to employ replication-competent viral vectors in monocot transformation. Such vectors include, for example, wheat dwarf virus (WDV) "shuttle" vectors, such as pW1-11 and PW1-GUS (Ugaki *et al.*, 1991). These vectors are capable of autonomous replication in maize cells as well as *E. coli*, and as such may provide increased sensitivity for detecting DNA delivered to transgenic cells. A replicating vector may also be useful for delivery of genes flanked by DNA sequences from transposable elements such as Ac, Ds, or Mu. It has been proposed (Laufs *et al.*, 1990) that transposition of these elements within the maize genome requires DNA replication. It is also contemplated that transposable elements would be useful for introducing DNA fragments lacking elements necessary for selection and maintenance of the plasmid vector in bacteria, e.g., antibiotic resistance genes and origins of DNA replication. It is also proposed that use of a transposable element such as Ac, Ds, or Mu would actively promote integration of the desired DNA and hence increase the frequency of stably transformed cells.

Vectors, plasmids, cosmids, YACs (yeast artificial chromosomes) and DNA segments for use in transforming such cells will, of course, generally comprise the cDNA, gene or genes which one desires to introduce into the cells. These DNA constructs can further include structures such as promoters, enhancers, polylinkers, or even regulatory genes as desired. The DNA segment or gene chosen for cellular introduction will often encode a protein which will be expressed

in the resultant recombinant cells, such as will result in a screenable or selectable trait and/or which will impart an improved phenotype to the regenerated plant. However, this may not always be the case, and the present invention also encompasses transgenic plants incorporating non-expressed transgenes.

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#### A. Regulatory Elements

The construction of vectors which may be employed in conjunction with the present invention will be known to those of skill of the art in light of the present disclosure (see e.g., Sambrook *et al.*, 1989; Gelvin *et al.*, 1990). Preferred constructs will generally include a plant promoter such as the CaMV 35S promoter (Odell *et al.*, 1985), or others such as CaMV 19S (Lawton *et al.*, 1987), *nos* (Ebert *et al.*, 1987), *Adh* (Walker *et al.*, 1987), sucrose synthase (Yang & Russell, 1990),  $\alpha$ -tubulin, actin (Wang *et al.*, 1992), *cab* (Sullivan *et al.*, 1989), PEPCase (Hudspeth & Grula, 1989) or those associated with the R gene complex (Chandler *et al.*, 1989). Tissue specific promoters such as root cell promoters (Conkling *et al.*, 1990) and tissue specific enhancers (Fromm *et al.*, 1989) are also contemplated to be particularly useful, as are inducible promoters such as ABA- and turgor-inducible promoters.

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Constructs will also include the gene of interest along with a 3' end DNA sequence that acts as a signal to terminate transcription and allow for the polyadenylation of the resultant mRNA. The most preferred 3' elements are contemplated to be those from the nopaline synthase gene of *Agrobacterium tumefaciens* (Bevan *et al.*, 1983), the terminator for the T7 transcript from the octopine synthase gene of *Agrobacterium tumefaciens*, and the 3' end of the protease inhibitor I or II genes from potato or tomato. Regulatory elements such as *Adh* intron 1 (Callis *et al.*, 1987), sucrose synthase intron (Vasil *et al.*, 1989) or TMV omega element (Gallie, *et al.*, 1989), may further be included where desired.

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As the DNA sequence between the transcription initiation site and the start of the coding sequence, i.e., the untranslated leader sequence, can influence gene expression, one may also wish to employ a particular leader sequence. Preferred leader sequences are contemplated to include those which include sequences predicted to direct optimum expression of the attached gene, i.e., to include a preferred consensus leader sequence which may increase or maintain mRNA stability and prevent inappropriate initiation of translation. The choice of such sequences will be known to those of skill in the art in light of the present disclosure. Sequences that are derived from genes that are highly expressed in plants, and in maize in particular, will be most preferred.

It is contemplated that vectors for use in accordance with the present invention may be constructed to include the *ocs* enhancer element. This element was first identified as a 16 bp palindromic enhancer from the octopine synthase (*ocs*) gene of agrobacterium (Ellis *et al.*, 1987), and is present in at least 10 other promoters (Bouchez *et al.*, 1989). It is proposed that the use of an enhancer element, such as the *ocs* element and particularly multiple copies of the element, will act to increase the level of transcription from adjacent promoters when applied in the context of monocot transformation.

Ultimately, the most desirable DNA segments for introduction into a monocot genome may be homologous genes or gene families which encode a desired trait (e.g., increased yield per acre) and which are introduced under the control of novel promoters or enhancers, etc., or perhaps even homologous or tissue specific (e.g., root-, collar/sheath-, whorl-, stalk-, earshank-, kernel- or leaf-specific) promoters or control elements. Indeed, it is envisioned that a particular use of the present invention will be the targeting of a gene in a tissue-specific manner. For example, insect resistant genes may be expressed specifically in the whorl and collar/sheath tissues which are targets for the first and second broods, respectively, of ECB. Likewise, genes encoding proteins with particular activity against rootworm may be



targeted directly to root tissues.

Vectors for use in tissue-specific targeting of genes in transgenic plants will typically include tissue-specific promoters and may also include other tissue-specific control elements such as enhancer sequences. Promoters which direct specific or enhanced expression in certain plant tissues will be known to those of skill in the art in light of the present disclosure. These include, for example, the *rbcS* promoter, specific for green tissue; the *ocs*, *nos* and *mas* promoters which have higher activity in roots or wounded leaf tissue; a truncated (-90 to +8) 35S promoter which directs enhanced expression in roots, an  $\alpha$ -tubulin gene that directs expression in roots and promoters derived from zein storage protein genes which direct expression in endosperm. It is particularly contemplated that one may advantageously use the 16 bp *ocs* enhancer element from the octopine synthase (*ocs*) gene (Ellis *et al.*, 1987; Bonchez *et al.*, 1989), especially when present in multiple copies, to achieve enhanced expression in roots.

It is also contemplated that tissue specific expression may be functionally accomplished by introducing a constitutively expressed gene (all tissues) in combination with an antisense gene that is expressed only in those tissues where the gene product is not desired. For example, a gene coding for the crystal toxin protein from *B. thuringiensis* (*Bt*) may be introduced such that it is expressed in all tissues using the 35S promoter from Cauliflower Mosaic Virus. Expression of an antisense transcript of the *Bt* gene in a maize kernel, using for example a zein promoter, would prevent accumulation of the *Bt* protein in seed. Hence the protein encoded by the introduced gene would be present in all tissues except the kernel.

Alternatively, one may wish to obtain novel tissue-specific promoter sequences for use in accordance with the present invention. To achieve this, one may first isolate cDNA clones from the tissue concerned and identify those clones

which are expressed specifically in that tissue, for example, using Northern blotting. Ideally, one would like to identify a gene that is not present in a high copy number, but which gene product is relatively abundant in specific tissues. The promoter and control elements of corresponding genomic clones may then be localized using the techniques of molecular biology known to those of skill in the art.

It is contemplated that expression of some genes in transgenic plants will be desired only under specified conditions. For example, it is proposed that expression of certain genes that confer resistance to environmental stress factors such as drought will be desired only under actual stress conditions. It is contemplated that expression of such genes throughout a plants development may have detrimental effects. It is known that a large number of genes exist that respond to the environment. For example, expression of some genes such as *rbcS*, encoding the small subunit of ribulose bisphosphate carboxylase, is regulated by light as mediated through phytochrome. Other genes are induced by secondary stimuli. For example, synthesis of abscisic acid (ABA) is induced by certain environmental factors, including but not limited to water stress. A number of genes have been shown to be induced by ABA (Skriver and Mundy, 1990). It is also anticipated that expression of genes conferring resistance to insect predation would be desired only under conditions of actual insect infestation. Therefore, for some desired traits inducible expression of genes in transgenic plants will be desired.

It is proposed that in some embodiments of the present invention expression of a gene in a transgenic plant will be desired only in a certain time period during the development of the plant. Developmental timing is frequently correlated with tissue specific gene expression. For example, expression of zein storage proteins is initiated in the endosperm about 15 days after pollination.

Additionally, vectors may be constructed and employed in the intracellular targeting of a specific gene product within the cells of a transgenic plant or in directing a protein to the extracellular environment. This will generally be achieved by joining a DNA sequence encoding a transit or signal peptide sequence to the coding sequence of a particular gene. The resultant transit, or signal, peptide will transport the protein to a particular intracellular, or extracellular destination, respectively, and will then be post-translationally removed. Transit or signal peptides act by facilitating the transport of proteins through intracellular membranes, e.g., vacuole, vesicle, plastid and mitochondrial membranes, whereas signal peptides direct proteins through the extracellular membrane.

A particular example of such a use concerns the direction of a herbicide resistance gene, such as the EPSPS gene, to a particular organelle such as the chloroplast rather than to the cytoplasm. This is exemplified by the use of the *rbcS* transit peptide which confers plastid-specific targeting of proteins. In addition, it is proposed that it may be desirable to target certain genes responsible for male sterility to the mitochondria, or to target certain genes for resistance to phytopathogenic organisms to the extracellular spaces, or to target proteins to the vacuole.

It is also contemplated that it may be useful to target DNA itself within a cell. For example, it may be useful to target introduced DNA to the nucleus as this may increase the frequency of transformation. Within the nucleus itself it would be useful to target a gene in order to achieve site specific integration. For example, it would be useful to have an gene introduced through transformation replace an existing gene in the cell.

## B. Marker Genes

In order to improve the ability to identify transformants, one may desire to employ a selectable or screenable marker gene as, or in addition to, the expressible gene of interest. "Marker genes" are genes that impart a distinct phenotype to cells expressing the marker gene and thus allow such transformed cells to be distinguished from cells that do not have the marker. Such genes may encode either a selectable or screenable marker, depending on whether the marker confers a trait which one can 'select' for by chemical means, i.e., through the use of a selective agent (e.g., a herbicide, antibiotic, or the like), or whether it is simply a trait that one can identify through observation or testing, i.e., by 'screening' (e.g., the R-locus trait). Of course, many examples of suitable marker genes are known to the art and can be employed in the practice of the invention.

Included within the terms selectable or screenable marker genes are also genes which encode a "secretable marker" whose secretion can be detected as a means of identifying or selecting for transformed cells. Examples include markers which encode a secretable antigen that can be identified by antibody interaction, or even secretable enzymes which can be detected by their catalytic activity.

Secretable proteins fall into a number of classes, including small, diffusible proteins detectable, e.g., by ELISA; small active enzymes detectable in extracellular solution (e.g.,  $\alpha$ -amylase,  $\beta$ -lactamase, phosphinothricin acetyltransferase); and proteins that are inserted or trapped in the cell wall (e.g., proteins that include a leader sequence such as that found in the expression unit of extensin or tobacco PR-S).

With regard to selectable secretable markers, the use of a gene that encodes a protein that becomes sequestered in the cell wall, and which protein includes a unique epitope is considered to be particularly advantageous. Such a secreted antigen marker would ideally employ an epitope sequence that would provide low background in plant tissue, a promoter-leader sequence that would impart efficient

expression and targeting across the plasma membrane, and would produce protein that is bound in the cell wall and yet accessible to antibodies. A normally secreted wall protein modified to include a unique epitope would satisfy all such requirements.

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One example of a protein suitable for modification in this manner is extensin, or hydroxyproline rich glycoprotein (HPRG). The use of the maize HPRG (Steifel *et al.*, 1990) which is preferred as this molecule is well characterized in terms of molecular biology, expression and protein structure. However, any one of a variety of extensins and/or glycine-rich wall proteins (Keller *et al.*, 1989) could be modified by the addition of an antigenic site to create a screenable marker.

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One exemplary embodiment of a secretable screenable marker concerns the use of the maize genomic clone encoding the wall protein HPRG, modified to include the unique 15 residue epitope M A T V P E L N C E M P P S D from the pro-region of murine interleukin-1- $\beta$  (IL-1- $\beta$ ). However, virtually any detectable epitope may be employed in such embodiments, as selected from the extremely wide variety of antigen:antibody combinations known to those of skill in the art. The unique extracellular epitope, whether derived from IL-1- $\beta$  or any other protein or epitopic substance, can then be straightforwardly detected using antibody labeling in conjunction with chromogenic or fluorescent adjuncts.

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Elements of the present disclosure are exemplified in detail through the use of the *bar* and/or GUS genes, and also through the use of various other markers. Of course, in light of this disclosure, numerous other possible selectable and/or screenable marker genes will be apparent to those of skill in the art in addition to the one set forth hereinbelow. Therefore, it will be understood that the following discussion is exemplary rather than exhaustive. In light of the techniques disclosed herein and the general recombinant techniques which are known in the art, the present invention renders possible the introduction of any gene, including marker

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genes, into a recipient cell to generate a transformed monocot.

## 1. Selectable Markers

Possible selectable markers for use in connection with the present invention include, but are not limited to, a *neo* gene (Potrykus *et al.*, 1985) which codes for kanamycin resistance and can be selected for using kanamycin, G418, etc.; a *bar* gene which codes for bialaphos resistance; a mutant *aroA* gene which encodes an altered EPSP synthase protein (Hinchee *et al.*, 1988) thus conferring glyphosate resistance; a nitrilase gene such as *bxn* from *Klebsiella ozaenae* which confers resistance to bromoxynil (Stalker *et al.*, 1988); a mutant acetolactate synthase gene (ALS) which confers resistance to imidazolinone, sulfonylurea or other ALS inhibiting chemicals (European Patent Application 154,204, 1985); a methotrexate resistant DHFR gene (Thillet *et al.*, 1988), or a dalapon dehalogenase gene that confers resistance to the herbicide dalapon; or a mutated anthranilate synthase gene that confers resistance to 5-methyl tryptophan. Where a mutant EPSP synthase gene is employed, additional benefit may be realized through the incorporation of a suitable chloroplast transit peptide, CTP (European Patent Application 0,218,571, 1987).

An illustrative embodiment of a selectable marker gene capable of being used in systems to select transformants is the genes that encode the enzyme phosphinothricin acetyltransferase, such as the *bar* gene from *Streptomyces hygroscopicus* or the *pat* gene from *Streptomyces viridochromogenes*. The enzyme phosphinothricin acetyl transferase (PAT) inactivates the active ingredient in the herbicide bialaphos, phosphinothricin (PPT). PPT inhibits glutamine synthetase, (Murakami *et al.*, 1986; Twell *et al.*, 1989) causing rapid accumulation of ammonia and cell death. The success of the inventors in using this selective system in conjunction with monocots was particularly surprising because of the major difficulties which have been reported in transformation of cereals (Potrykus, 1989).

Where one desires to employ a bialaphos resistance gene in the practice of the invention, the inventors have discovered that a particularly useful gene for this purpose is the *bar* or *pat* genes obtainable from species of *Streptomyces* (e.g., ATCC No. 21,705). The cloning of the *bar* gene has been described (Murakami *et al.*, 1986; Thompson *et al.*, 1987) as has the use of the *bar* gene in the context of plants other than monocots (De Block *et al.*, 1987; De Block *et al.*, 1989).

## 2. Screenable Markers

Screenable markers that may be employed include a  $\beta$ -glucuronidase or *uidA* gene (GUS) which encodes an enzyme for which various chromogenic substrates are known; an R-locus gene, which encodes a product that regulates the production of anthocyanin pigments (red color) in plant tissues (Dellaporta *et al.*, 1988); a  $\beta$ -lactamase gene (Sutcliffe, 1978), which encodes an enzyme for which various chromogenic substrates are known (e.g., PADAC, a chromogenic cephalosporin); a *xy/E* gene (Zukowsky *et al.*, 1983) which encodes a catechol dioxygenase that can convert chromogenic catechols; an  $\alpha$ -amylase gene (Ikuta *et al.*, 1990); a tyrosinase gene (Katz *et al.*, 1983) which encodes an enzyme capable of oxidizing tyrosine to DOPA and dopaquinone which in turn condenses to form the easily-detectable compound melanin; a  $\beta$ -galactosidase gene, which encodes an enzyme for which there are chromogenic substrates; a luciferase (*lux*) gene (Ow *et al.*, 1986), which allows for bioluminescence detection; or even an aequorin gene (Prasher *et al.*, 1985) which may be employed in calcium-sensitive bioluminescence detection.

Genes from the maize R gene complex are contemplated to be particularly useful as screenable markers. The R gene complex in maize encodes a protein that acts to regulate the production of anthocyanin pigments in most seed and plant tissue. Maize strains can have one, or as many as four, R alleles which combine to regulate pigmentation in a developmental and tissue specific manner. The present inventors have applied a gene from the R gene complex to maize transformation, because the

expression of this gene in transformed cells does not harm the cells. Thus, an R gene introduced into such cells will cause the expression of a red pigment and, if stably incorporated, can be visually scored as a red sector. If a maize line carries dominant alleles for genes encoding the enzymatic intermediates in the anthocyanin biosynthetic pathway (C2, A1, A2, Bz1 and Bz2), but carries a recessive allele at the R locus, transformation of any cell from that line with R will result in red pigment formation. Exemplary lines include Wisconsin 22 which contains the rg-Stadler allele and TR112, a K55 derivative which is r-g, b, Pl. Alternatively any genotype of maize can be utilized if the C1 and R alleles are introduced together.

The inventors further propose that R gene regulatory regions may be employed in chimeric constructs in order to provide mechanisms for controlling the expression of chimeric genes. More diversity of phenotypic expression is known at the R locus than at any other locus (Coe *et al.*, 1988). It is contemplated that regulatory regions obtained from regions 5' to the structural R gene would be valuable in directing the expression of genes for, e.g., insect resistance, herbicide tolerance or other protein coding regions. For the purposes of the present invention, it is believed that any of the various R gene family members may be successfully employed (e.g., P, S, Lc, etc.). However, the most preferred will generally be Sn (particularly Sn:bol3). Sn is a dominant member of the R gene complex and is functionally similar to the R and B loci in that Sn controls the tissue specific deposition of anthocyanin pigments in certain seedling and plant cells, therefore, its phenotype is similar to R.

A further screenable marker contemplated for use in the present invention is firefly luciferase, encoded by the *lux* gene. The presence of the *lux* gene in transformed cells may be detected using, for example, X-ray film, scintillation counting, fluorescent spectrophotometry, low-light video cameras, photon counting cameras or multiwell luminometry. It is also envisioned that this system may be developed for populational screening for bioluminescence, such as on tissue culture plates, or even for whole plant screening.



### C. Transgenes for Corn Modification

A particularly important advance of the present invention is that it provides methods and compositions for the transformation of plant cells with genes in addition to, or other than, marker genes. Such transgenes will often be genes that direct the expression of a particular protein or polypeptide product, but they may also be non-expressible DNA segments, e.g., transposons such as Ds that do not direct their own transposition. As used herein, an "expressible gene" is any gene that is capable of being transcribed into RNA (e.g., mRNA, antisense RNA, etc.) or translated into a protein, expressed as a trait of interest, or the like, etc., and is not limited to selectable, screenable or non-selectable marker genes. The invention also contemplates that, where both an expressible gene that is not necessarily a marker gene is employed in combination with a marker gene, one may employ the separate genes on either the same or different DNA segments for transformation. In the latter case, the different vectors are delivered concurrently to recipient cells to maximize cotransformation.

The choice of the particular DNA segments to be delivered to the recipient cells will often depend on the purpose of the transformation. One of the major purposes of transformation of crop plants is to add some commercially desirable, agronomically important traits to the plant. Such traits include, but are not limited to, herbicide resistance or tolerance; insect resistance or tolerance; disease resistance or tolerance (viral, bacterial, fungal, nematode); stress tolerance and/or resistance, as exemplified by resistance or tolerance to drought, heat, chilling, freezing, excessive moisture, salt stress; oxidative stress; increased yields; food content and makeup; physical appearance; male sterility; drydown; standability; prolificacy; starch properties; oil quantity and quality; and the like. One may desire to incorporate one or more genes conferring any such desirable trait or traits, such as, for example, a gene or genes encoding herbicide resistance.

In certain embodiments, the present invention contemplates the transformation of a recipient cell with more than one advantageous transgene. Two or more transgenes can be supplied in a single transformation event using either distinct transgene-encoding vectors, or using a single vector incorporating two or more gene coding sequences. For example, plasmids bearing the *bar* and *aroA* expression units in either convergent, divergent, or colinear orientation, are considered to be particularly useful. Further preferred combinations are those of an insect resistance gene, such as a Bt gene, along with a protease inhibitor gene such as *pinII*, or the use of *bar* in combination with either of the above genes. Of course, any two or more transgenes of any description, such as those conferring herbicide, insect, disease (viral, bacterial, fungal, nematode) or drought resistance, male sterility, drydown, standability, prolificacy, starch properties, oil quantity and quality, or those increasing yield or nutritional quality may be employed as desired.

## 1. Herbicide Resistance

The genes encoding phosphinothricin acetyltransferase (*bar* and *pat*), glyphosate tolerant EPSP synthase genes, the glyphosate degradative enzyme gene *gox* encoding glyphosate oxidoreductase, *deh* (encoding a dehalogenase enzyme that inactivates dalapon), herbicide resistant (e.g., sulfonylurea and imidazolinone) acetolactate synthase, and *bxn* genes (encoding a nitrilase enzyme that degrades bromoxynil) are good examples of herbicide resistant genes for use in transformation. The *bar* and *pat* genes code for an enzyme, phosphinothricin acetyltransferase (PAT), which inactivates the herbicide phosphinothricin and prevents this compound from inhibiting glutamine synthetase enzymes. The enzyme 5-enolpyruvylshikimate 3-phosphate synthase (EPSP Synthase), is normally inhibited by the herbicide N-(phosphonomethyl)glycine (glyphosate). However, genes are known that encode glyphosate-resistant EPSP Synthase enzymes. These genes are particularly contemplated for use in monocot transformation. The *deh* gene encodes the enzyme dalapon dehalogenase and confers resistance to the herbicide dalapon. The *bxn* gene

codes for a specific nitrilase enzyme that converts bromoxynil to a non-herbicidal degradation product.

## 2. Insect Resistance

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An important aspect of the present invention concerns the introduction of insect resistance-conferring genes into monocotyledonous plants such as maize. Potential insect resistance genes which can be introduced include *Bacillus thuringiensis* crystal toxin genes or Bt genes (Watrud *et al.*, 1985). Bt genes may provide resistance to  
10 lepidopteran or coleopteran pests such as European Corn Borer (ECB). Preferred Bt toxin genes for use in such embodiments include the *CryIA(b)* and *CryIA(c)* genes. Endotoxin genes from other species of *B. thuringiensis* which affect insect growth or development may also be employed in this regard.

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The poor expression of procaryotic Bt toxin genes in plants is a well-documented phenomenon, and the use of different promoters, fusion proteins, and leader sequences has not led to significant increases in Bt protein expression (Vaeck *et al.*, 1989; Barton *et al.*, 1987). It is therefore contemplated that the most advantageous Bt genes for use in the transformation protocols disclosed herein will  
20 be those in which the coding sequence has been modified to effect increased expression in plants, and more particularly, those in which maize preferred codons have been used. Examples of such modified Bt toxin genes include the variant Bt *CryIA(b)* gene termed *IAb6* (Perlak *et al.*, 1991) and the synthetic *CryIA(c)* genes termed 1800a and 1800b.

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Protease inhibitors may also provide insect resistance (Johnson *et al.*, 1989), and will thus have utility in maize transformation. The use of a protease inhibitor II gene, *pinII*, from tomato or potato is envisioned to be particularly useful. Even more advantageous is the use of a *pinII* gene in combination with a Bt toxin gene, the  
30 combined effect of which has been discovered by the present inventors to produce

synergistic insecticidal activity. Other genes which encode inhibitors of the insects' digestive system, or those that encode enzymes or co-factors that facilitate the production of inhibitors, may also be useful. This group may be exemplified by oryzacystatin and amylase inhibitors such as those from wheat and barley.

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Also, genes encoding lectins may confer additional or alternative insecticide properties. Lectins (originally termed phytohemagglutinins) are multivalent carbohydrate-binding proteins which have the ability to agglutinate red blood cells from a range of species. Lectins have been identified recently as insecticidal agents with activity against weevils, ECB and rootworm (Murdock *et al.*, 1990; Czapla & Lang, 1990). Lectin genes contemplated to be useful include, for example, barley and wheat germ agglutinin (WGA) and rice lectins (Gatehouse *et al.*, 1984), with WGA being preferred.

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Genes controlling the production of large or small polypeptides active against insects when introduced into the insect pests, such as, e.g., lytic peptides, peptide hormones and toxins and venoms, form another aspect of the invention. For example, it is contemplated that the expression of juvenile hormone esterase, directed towards specific insect pests, may also result in insecticidal activity, or perhaps cause cessation of metamorphosis (Hammock *et al.*, 1990).

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Transgenic maize expressing genes which encode enzymes that affect the integrity of the insect cuticle form yet another aspect of the invention. Such genes include those encoding, e.g., chitinase, proteases, lipases and also genes for the production of nikkomycin, a compound that inhibits chitin synthesis, the introduction of any of which is contemplated to produce insect resistant maize plants. Genes that code for activities that affect insect molting, such those affecting the production of ecdysteroid UDP-glucosyl transferase, also fall within the scope of the useful transgenes of the present invention.

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Genes that code for enzymes that facilitate the production of compounds that reduce the nutritional quality of the host plant to insect pests are also encompassed by the present invention. It may be possible, for instance, to confer insecticidal activity on a plant by altering its sterol composition. Sterols are obtained by insects from their diet and are used for hormone synthesis and membrane stability. Therefore alterations in plant sterol composition by expression of novel genes, e.g., those that directly promote the production of undesirable sterols or those that convert desirable sterols into undesirable forms, could have a negative effect on insect growth and/or development and hence endow the plant with insecticidal activity. Lipoxygenases are naturally occurring plant enzymes that have been shown to exhibit anti-nutritional effects on insects and to reduce the nutritional quality of their diet. Therefore, further embodiments of the invention concern transgenic plants with enhanced lipoxygenase activity which may be resistant to insect feeding.

The present invention also provides methods and compositions by which to achieve qualitative or quantitative changes in plant secondary metabolites. One example concerns transforming maize to produce DIMBOA which, it is contemplated, will confer resistance to European corn borer, rootworm and several other maize insect pests. Candidate genes that are particularly considered for use in this regard include those genes at the bx locus known to be involved in the synthetic DIMBOA pathway (Dunn *et al.*, 1981). The introduction of genes that can regulate the production of maysin, and genes involved in the production of dhurrin in sorghum, is also contemplated to be of use in facilitating resistance to earworm and rootworm, respectively.

*Tripsacum dactyloides* is a species of grass that is resistant to certain insects, including corn root worm. It is anticipated that genes encoding proteins that are toxic to insects or are involved in the biosynthesis of compounds toxic to insects will be isolated from *Tripsacum* and that these novel genes will be useful in conferring resistance to insects. It is known that the basis of insect resistance in *Tripsacum* is

genetic, because said resistance has been transferred to *Zea mays* via sexual crosses (Branson and Guss, 1972).

Further genes encoding proteins characterized as having potential insecticidal activity may also be used as transgenes in accordance herewith. Such genes include, for example, the cowpea trypsin inhibitor (CpTI; Hilder *et al.*, 1987) which may be used as a rootworm deterrent; genes encoding avermectin (*Avermectin and Abamectin.*, Campbell, W.C., Ed., 1989; Ikeda *et al.*, 1987) which may prove particularly useful as a corn rootworm deterrent; ribosome inactivating protein genes; and even genes that regulate plant structures. Transgenic maize including anti-insect antibody genes and genes that code for enzymes that can covert a non-toxic insecticide (pro-insecticide) applied to the outside of the plant into an insecticide inside the plant are also contemplated.

### 3. Environment or Stress Resistance

Improvement of corn's ability to tolerate various environmental stresses such as, but not limited to, drought, excess moisture, chilling, freezing, high temperature, salt, and oxidative stress, can also be effected through expression of novel genes. It is proposed that benefits may be realized in terms of increased resistance to freezing temperatures through the introduction of an "antifreeze" protein such as that of the Winter Flounder (Cutler *et al.*, 1989) or synthetic gene derivatives thereof. Improved chilling tolerance may also be conferred through increased expression of glycerol-3-phosphate acetyltransferase in chloroplasts (Murata *et al.*, 1992; Wolter *et al.*, 1992). Resistance to oxidative stress (often exacerbated by conditions such as chilling temperatures in combination with high light intensities) can be conferred by expression of superoxide dismutase (Gupta *et al.*, 1993), and may be improved by glutathione reductase (Bowler *et al.*, 1992). Such strategies may allow for tolerance to freezing in newly emerged fields as well as extending later maturity higher yielding varieties to earlier relative maturity

zones.

It is contemplated that the expression of novel genes that favorably effect plant water content, total water potential, osmotic potential, and turgor will enhance the ability of the plant to tolerate drought. As used herein, the terms "drought resistance" and "drought tolerance" are used to refer to a plants increased resistance or tolerance to stress induced by a reduction in water availability, as compared to normal circumstances, and the ability of the plant to function and survive in lower-water environments. In this aspect of the invention it is proposed, for example, that the expression of genes encoding for the biosynthesis of osmotically-active solutes, such as polyol compounds, may impart protection against drought. Within this class are genes encoding for mannitol dehydrogenase (Lee and Saier, 1982) and trehalose-6-phosphate synthase (Kaasen et al., 1992). Through the subsequent action of native phosphatases in the cell or by the introduction and coexpression of a specific phosphatase, these introduced genes will result in the accumulation of either mannitol or trehalose, respectively, both of which have been well documented as protective compounds able to mitigate the effects of stress. Mannitol accumulation in transgenic tobacco has been verified and preliminary results indicate that plants expressing high levels of this metabolite are able to tolerate an applied osmotic stress (Tarczynski et al., 1992, 1993).

Similarly, the efficacy of other metabolites in protecting either enzyme function (e.g. alanopine or propionic acid) or membrane integrity (e.g., alanopine) has been documented (Loomis et al., 1989), and therefore expression of genes encoding for the biosynthesis of these compounds might confer drought resistance in a manner similar to or complimentary to mannitol. Other examples of naturally occurring metabolites that are osmotically active and/or provide some direct protective effect during drought and/or desiccation include fructose, erythritol (Coxson et al., 1992), sorbitol, dulcitol (Karsten et al., 1992), glucosylglycerol

(Reed et al., 1984; Erdmann et al., 1992), sucrose, stachyose (Koster and Leopold, 1988; Blackman et al., 1992), raffinose (Bernal-Lugo and Leopold, 1992), proline (Rensburg et al., 1993) and glycinebetaine (Wyn-Jones and Storey, 1982), ononitol and pinitol (Vernon and Bohnert, 1992). Continued canopy growth and increased reproductive fitness during times of stress will be augmented by introduction and expression of genes such as those controlling the osmotically active compounds discussed above and other such compounds. Currently preferred genes which promote the synthesis of an osmotically active polyol compound are genes which encode the enzymes mannitol-1-phosphate dehydrogenase, trehalose-6-phosphate synthase and myoinositol O-methyltransferase.

It is contemplated that the expression of specific proteins may also increase drought tolerance. Three classes of Late Embryogenic Proteins have been assigned based on structural similarities (see Dure et al., 1989). All three classes of LEAs have been demonstrated in maturing (i.e. desiccating) seeds. Within these 3 types of LEA proteins, the Type-II (dehydrin-type) have generally been implicated in drought and/or desiccation tolerance in vegetative plant parts (i.e. Mundy and Chua, 1988; Piatkowski et al., 1990; Yamaguchi-Shinozaki et al., 1992). Recently, expression of a Type-III LEA (HVA-1) in tobacco was found to influence plant height, maturity and drought tolerance (Fitzpatrick, 1993). Expression of structural genes from all three LEA groups may therefore confer drought tolerance. Other types of proteins induced during water stress include thiol proteases, aldolases and transmembrane transporters (Guerrero et al., 1990), which may confer various protective and/or repair-type functions during drought stress. It is also contemplated that genes that effect lipid biosynthesis and hence membrane composition might also be useful in conferring drought resistance on the plant.

Many of these genes for improving drought resistance have complementary modes of action. Thus, it is envisaged that combinations of these genes might have additive and/or synergistic effects in improving drought resistance in corn.



Many of these genes also improve freezing tolerance (or resistance); the physical stresses incurred during freezing and drought are similar in nature and may be mitigated in similar fashion. Benefit may be conferred via constitutive expression of these genes, but the preferred means of expressing these novel genes may be through the use of a turgor-induced promoter (such as the promoters for the turgor-induced genes described in Guerrero et al., 1987 and Shagan et al., 1993 which are incorporated herein by reference). Spatial and temporal expression patterns of these genes may enable corn to better withstand stress.

It is proposed that expression of genes that are involved with specific morphological traits that allow for increased water extractions from drying soil would be of benefit. For example, introduction and expression of genes that alter root characteristics may enhance water uptake. It is also contemplated that expression of genes that enhance reproductive fitness during times of stress would be of significant value. For example, expression of genes that improve the synchrony of pollen shed and receptiveness of the female flower parts, i.e., silks, would be of benefit. In addition it is proposed that expression of genes that minimize kernel abortion during times of stress would increase the amount of grain to be harvested and hence be of value.

Given the overall role of water in determining yield, it is contemplated that enabling corn to utilize water more efficiently, through the introduction and expression of novel genes, will improve overall performance even when soil water availability is not limiting. By introducing genes that improve the ability of corn to maximize water usage across a full range of stresses relating to water availability, yield stability or consistency of yield performance may be realized.

#### 4. Disease Resistance

It is proposed that increased resistance to diseases may be realized through introduction of genes into monocotyledonous plants such as maize. It is possible to produce resistance to diseases caused by viruses, bacteria, fungi and nematodes. It is also contemplated that control of mycotoxin producing organisms may be realized through expression of introduced genes.

Resistance to viruses may be produced through expression of novel genes. For example, it has been demonstrated that expression of a viral coat protein in a transgenic plant can impart resistance to infection of the plant by that virus and perhaps other closely related viruses (Cuozzo et al., 1988, Hemenway et al., 1988, Abel et al., 1986). It is contemplated that expression of antisense genes targeted at essential viral functions may impart resistance to said virus. For example, an antisense gene targeted at the gene responsible for replication of viral nucleic acid may inhibit said replication and lead to resistance to the virus. It is believed that interference with other viral functions through the use of antisense genes may also increase resistance to viruses. Further it is proposed that it may be possible to achieve resistance to viruses through other approaches, including, but not limited to the use of satellite viruses.

It is proposed that increased resistance to diseases caused by bacteria and fungi may be realized through introduction of novel genes. It is contemplated that genes encoding so-called "peptide antibiotics," pathogenesis related (PR) proteins, toxin resistance, and proteins affecting host-pathogen interactions such as morphological characteristics will be useful. Peptide antibiotics are polypeptide sequences which are inhibitory to growth of bacteria and other microorganisms. For example, the classes of peptides referred to as cecropins and magainins inhibit growth of many species of bacteria and fungi. It is proposed that expression of PR proteins in monocotyledonous plants such as maize may be useful in conferring

resistance to bacterial disease. These genes are induced following pathogen attack on a host plant and have been divided into at least five classes of proteins (Bol, Linthorst, and Cornelissen, 1990). Included amongst the PR proteins are  $\beta$ -1, 3-glucanases, chitinases, and osmotin and other proteins that are believed to function in plant resistance to disease organisms. Other genes have been identified that have antifungal properties, e.g., UDA (stinging nettle lectin) and hevein (Broekaert *et al.*, 1989; Barkai-Golan *et al.*, 1978). It is known that certain plant diseases are caused by the production of phytotoxins. It is proposed that resistance to these diseases would be achieved through expression of a novel gene that encodes an enzyme capable of degrading or otherwise inactivating the phytotoxin. It is also contemplated that expression novel genes that alter the interactions between the host plant and pathogen may be useful in reducing the ability the disease organism to invade the tissues of the host plant, e.g., an increase in the waxiness of the leaf cuticle or other morphological characteristics.

Plant parasitic nematodes are a cause of disease in many plants, including maize. It is proposed that it would be possible to make the corn plant resistant to these organisms through the expression of novel genes. It is anticipated that control of nematode infestations would be accomplished by altering the ability of the nematode to recognize or attach to a host plant and/or enabling the plant to produce nematicidal compounds, including but not limited to proteins.

## **5. Mycotoxin Reduction/Elimination**

Production of mycotoxins, including aflatoxin and fumonisin, by fungi associated with monocotyledonous plants such as maize is a significant factor in rendering the grain not useful. These fungal organisms do not cause disease symptoms and/or interfere with the growth of the plant, but they produce chemicals (mycotoxins) that are toxic to animals. It is contemplated that inhibition of the growth of these fungi would be reduce the synthesis of these toxic

substances and therefore reduce grain losses due to mycotoxin contamination. It is also proposed that it may be possible to introduce novel genes into monocotyledonous plants such as maize that would inhibit synthesis of the mycotoxin without interfering with fungal growth. Further, it is contemplated that expression of a novel gene which encodes an enzyme capable of rendering the mycotoxin nontoxic would be useful in order to achieve reduced mycotoxin contamination of grain. The result of any of the above mechanisms would be a reduced presence of mycotoxins on grain.

## 6. Grain Composition or Quality

Genes may be introduced into monocotyledonous plants, particularly commercially important cereals such as maize, to improve the grain for which the cereal is primarily grown. A wide range of novel transgenic plants produced in this manner may be envisioned depending on the particular end use of the grain.

The largest use of maize grain is for feed or food. Introduction of genes that alter the composition of the grain may greatly enhance the feed or food value. The primary components of maize grain are starch, protein, and oil. Each of these primary components of maize grain may be improved by altering its level or composition. Several examples may be mentioned for illustrative purposes but in no way provide an exhaustive list of possibilities.

The protein of cereal grains including maize is suboptimal for feed and food purposes especially when fed to pigs, poultry, and humans. The protein is deficient in several amino acids that are essential in the diet of these species, requiring the addition of supplements to the grain. Limiting essential amino acids may include lysine, methionine, tryptophan, threonine, valine, arginine, and histidine. Some amino acids become limiting only after corn is supplemented with other inputs for feed formulations. For example, when corn is supplemented with

soybean meal to meet lysine requirements methionine becomes limiting. The levels of these essential amino acids in seeds and grain may be elevated by mechanisms which include, but are not limited to, the introduction of genes to increase the biosynthesis of the amino acids, decrease the degradation of the amino acids, increase the storage of the amino acids in proteins, , or increase transport of the amino acids to the seeds or grain.

One mechanism for increasing the biosynthesis of the amino acids is to introduce genes that deregulate the amino acid biosynthetic pathways such that the plant can no longer adequately control the levels that are produced. This may be done by deregulating or bypassing steps in the amino acid biosynthetic pathway which are normally regulated by levels of the amino acid end product of the pathway. Examples include the introduction of genes that encode deregulated versions of the enzymes aspartokinase or dihydrodipicolinic acid (DHDP)-synthase for increasing lysine and threonine production, and anthranilate synthase for increasing tryptophan production. Reduction of the catabolism of the amino acids may be accomplished by introduction of DNA sequences that reduce or eliminate the expression of genes encoding enzymes that catalyze steps in the catabolic pathways such as the enzyme lysine-ketoglutarate reductase.

The protein composition of the grain may be altered to improve the balance of amino acids in a variety of ways including elevating expression of native proteins, decreasing expression of those with poor composition, changing the composition of native proteins, or introducing genes encoding entirely new proteins possessing superior composition. Examples may include the introduction of DNA that decreases the expression of members of the zein family of storage proteins. This DNA may encode ribozymes or antisense sequences directed to impairing expression of zein proteins or expression of regulators of zein expression such as the opaque-2 gene product. It is also proposed that the protein composition of the grain may be modified through the phenomenon of cosuppression, i.e., inhibition of

expression of an endogenous gene through the expression of an identical structural gene or gene fragment introduced through transformation (Goring et al., 1991).

Additionally, the introduced DNA may encode enzymes which degrade zeins. The decreases in zein expression that are achieved may be accompanied by increases in proteins with more desirable amino acid composition or increases in other major seed constituents such as starch. Alternatively, a chimeric gene may be introduced that comprises a coding sequence for a native protein of adequate amino acid composition such as for one of the globulin proteins or 10 kD zein of maize and a promoter or other regulatory sequence designed to elevate expression of said protein. The coding sequence of said gene may include additional or replacement codons for essential amino acids. Further, a coding sequence obtained from another species, or, a partially or completely synthetic sequence encoding a completely unique peptide sequence designed to enhance the amino acid composition of the seed may be employed.

The introduction of genes that alter the oil content of the grain may be of value. Increases in oil content may result in increases in metabolizable-energy-content and -density of the seeds for uses in feed and food. The introduced genes may encode enzymes that remove or reduce rate-limitations or regulated steps in fatty acid or lipid biosynthesis. Such genes may include, but are not limited to, those that encode acetyl-CoA carboxylase, ACP-acyltransferase,  $\beta$ -ketoacyl-ACP synthase, plus other well known fatty acid biosynthetic activities. Other possibilities are genes that encode proteins that do not possess enzymatic activity such as acyl carrier protein. Genes may be introduced that alter the balance of fatty acids present in the oil providing a more healthful or nutritive feedstuff. The introduced DNA may also encode sequences that block expression of enzymes involved in fatty acid biosynthesis, altering the proportions of fatty acids present in the grain such as described below.

Genes may be introduced that enhance the nutritive value of the starch

component of the grain, for example by increasing the degree of branching, resulting in improved utilization of the starch in cows by delaying its metabolism.

Besides affecting the major constituents of the grain, genes may be introduced that affect a variety of other nutritive, processing, or other quality aspects of the grain as used for feed or food. For example, pigmentation of the grain may be increased or decreased. Enhancement and stability of yellow pigmentation is desirable in some animal feeds and may be achieved by introduction of genes that result in enhanced production of xanthophylls and carotenes by eliminating rate-limiting steps in their production. Such genes may encode altered forms of the enzymes phytoene synthase, phytoene desaturase, or lycopene synthase. Alternatively, unpigmented white corn is desirable for production of many food products and may be produced by the introduction of DNA which blocks or eliminates steps in pigment production pathways.

Feed or food comprising primarily maize or other cereal grains possesses insufficient quantities of vitamins and must be supplemented to provide adequate nutritive value. Introduction of genes that enhance vitamin biosynthesis in seeds may be envisioned including, for example, vitamins A, E, B<sub>12</sub>, choline, and the like. Maize grain also does not possess sufficient mineral content for optimal nutritive value. Genes that affect the accumulation or availability of compounds containing phosphorus, sulfur, calcium, manganese, zinc, and iron among others would be valuable. An example may be the introduction of a gene that reduced phytic acid production or encoded the enzyme phytase which enhances phytic acid breakdown. These genes would increase levels of available phosphate in the diet, reducing the need for supplementation with mineral phosphate.

Numerous other examples of improvement of maize or other cereals for feed and food purposes might be described. The improvements may not even necessarily involve the grain, but may, for example, improve the value of the corn

for silage. Introduction of DNA to accomplish this might include sequences that alter lignin production such as those that result in the "brown midrib" phenotype associated with superior feed value for cattle.

5           In addition to direct improvements in feed or food value, genes may also be introduced which improve the processing of corn and improve the value of the products resulting from the processing. The primary method of processing corn is via wetmilling. Maize may be improved though the expression of novel genes that increase the efficiency and reduce the cost of processing such as by decreasing  
10           steeping time.

          Improving the value of wetmilling products may include altering the quantity or quality of starch, oil, corn gluten meal, or the components of corn gluten feed. Elevation of starch may be achieved through the identification and elimination of  
15           rate limiting steps in starch biosynthesis or by decreasing levels of the other components of the grain resulting in proportional increases in starch. An example of the former may be the introduction of genes encoding ADP-glucose pyrophosphorylase enzymes with altered regulatory activity or which are expressed at higher level. Examples of the latter may include selective inhibitors of, for  
20           example, protein or oil biosynthesis expressed during later stages of kernel development.

          The properties of starch may be beneficially altered by changing the ratio of amylose to amylopectin, the size of the starch molecules, or their branching  
25           pattern. Through these changes a broad range of properties may be modified which include, but are not limited to, changes in gelatinization temperature, heat of gelatinization, clarity of films and pastes, rheological properties, and the like. To accomplish these changes in properties, genes that encode granule-bound or soluble starch synthase activity or branching enzyme activity may be introduced  
30           alone or combination. DNA such as antisense constructs may also be used to



decrease levels of endogenous activity of these enzymes. The introduced genes or constructs may possess regulatory sequences that time their expression to specific intervals in starch biosynthesis and starch granule development. Furthermore, it may be worthwhile to introduce and express genes that result in the *in vivo* derivatization, or other modification, of the glucose moieties of the starch molecule. The covalent attachment of any molecule may be envisioned, limited only by the existence of enzymes that catalyze the derivatizations and the accessibility of appropriate substrates in the starch granule. Examples of important derivations may include the addition of functional groups such as amines, carboxyls, or phosphate groups which provide sites for subsequent *in vitro* derivatizations or affect starch properties through the introduction of ionic charges. Examples of other modifications may include direct changes of the glucose units such as loss of hydroxyl groups or their oxidation to aldehyde or carboxyl groups.

Oil is another product of wetmilling of corn, the value of which may be improved by introduction and expression of genes. The quantity of oil that can be extracted by wetmilling may be elevated by approaches as described for feed and food above. Oil properties may also be altered to improve its performance in the production and use of cooking oil, shortenings, lubricants or other oil-derived products or improvement of its health attributes when used in the food-related applications. Novel fatty acids may also be synthesized which upon extraction can serve as starting materials for chemical syntheses. The changes in oil properties may be achieved by altering the type, level, or lipid arrangement of the fatty acids present in the oil. This in turn may be accomplished by the addition of genes that encode enzymes that catalyze the synthesis of novel fatty acids and the lipids possessing them or by increasing levels of native fatty acids while possibly reducing levels of precursors. Alternatively DNA sequences may be introduced which slow or block steps in fatty acid biosynthesis resulting in the increase in precursor fatty acid intermediates. Genes that might be added include desaturases, epoxidases, hydratases, dehydratases, and other enzymes that catalyze reactions

involving fatty acid intermediates. Representative examples of catalytic steps that might be blocked include the desaturations from stearic to oleic acid and oleic to linolenic acid resulting in the respective accumulations of stearic and oleic acids. Another example is the blockage of elongation steps resulting in the accumulation of  $c_8$  to  $c_{12}$  saturated fatty acids.

Improvements in the other major corn wetmilling products, corn gluten meal and corn gluten feed, may also be achieved by the introduction of genes to obtain novel corn plants. Representative possibilities include but are not limited to those described above for improvement of food and feed value.

In addition it may further be considered that the corn plant be used for the production or manufacturing of useful biological compounds that were either not produced at all, or not produced at the same level, in the corn plant previously. The novel corn plants producing these compounds are made possible by the introduction and expression of genes by corn transformation methods. The vast array of possibilities include but are not limited to any biological compound which is presently produced by any organism such as proteins, nucleic acids, primary and intermediary metabolites, carbohydrate polymers, etc. The compounds may be produced by the plant, extracted upon harvest and/or processing, and used for any presently recognized useful purpose such as pharmaceuticals, fragrances, industrial enzymes to name a few.

Further possibilities to exemplify the range of grain traits or properties potentially encoded by introduced genes in transgenic plants include grain with less breakage susceptibility for export purposes or larger grit size when processed by dry milling through introduction of genes that enhance  $\gamma$ -zein synthesis, popcorn with improved popping quality and expansion volume through genes that increase pericarp thickness, corn with whiter grain for food uses through introduction of genes that effectively block expression of enzymes involved in pigment production

pathways, and improved quality of alcoholic beverages or sweet corn through introduction of genes which affect flavor such as the shrunken gene (encoding sucrose synthase) for sweet corn.

## 7. Plant Agronomic Characteristics

Two of the factors determining where corn can be grown are the average daily temperature during the growing season and the length of time between frosts.

5 Within the areas where it is possible to grow corn, there are varying limitations on the maximal time it is allowed to grow to maturity and be harvested. The corn to be grown in a particular area is selected for its ability to mature and dry down to harvestable moisture content within the required period of time with maximum possible yield. Therefore, corn of varying maturities is developed for different  
10 growing locations. Apart from the need to dry down sufficiently to permit harvest is the desirability of having maximal drying take place in the field to minimize the amount of energy required for additional drying post-harvest. Also the more readily the grain can dry down, the more time there is available for growth and kernel fill. It is considered that genes that influence maturity and/or dry down can be  
15 identified and introduced into corn lines using transformation techniques to create new corn varieties adapted to different growing locations or the same growing location but having improved yield to moisture ratio at harvest. Expression of genes that are involved in regulation of plant development may be especially useful, e.g., the liguleless and rough sheath genes that have been identified in  
20 corn.

It is contemplated that genes may be introduced into corn that would improve standability and other plant growth characteristics. Expression of novel genes which confer stronger stalks, improved root systems, or prevent or reduce  
25 ear droppage would be of great value to the farmer. It is proposed that introduction and expression of genes that increase the total amount of photoassimilate available by, for example, increasing light distribution and/or interception would be advantageous. In addition the expression of genes that increase the efficiency of photosynthesis and/or the leaf canopy would further  
30 increase gains in productivity. Such approaches would allow for increased plant

populations in the field.

Delay of late season vegetative senescence would increase the flow of  
assimilate into the grain and thus increase yield. It is proposed that overexpression  
of genes within corn that are associated with "stay green" or the expression of  
any gene that delays senescence would achieve be advantageous. For example, a  
nonyellowing mutant has been identified in *Festuca pratensis* (Davies et al., 1990).  
Expression of this gene as well as others may prevent premature breakdown of  
chlorophyll and thus maintain canopy function.

## 8. Nutrient Utilization

The ability to utilize available nutrients may be a limiting factor in growth of  
monocotyledonous plants such as maize. It is proposed that it would be possible  
to alter nutrient uptake, tolerate pH extremes, mobilization through the plant,  
storage pools, and availability for metabolic activities by the introduction of novel  
genes. These modifications would allow a plant such as maize to more efficiently  
utilize available nutrients. It is contemplated that an increase in the activity of, for  
example, an enzyme that is normally present in the plant and involved in nutrient  
utilization would increase the availability of a nutrient. An example of such an  
enzyme would be phytase. It is also contemplated that expression of a novel gene  
may make a nutrient source available that was previously not accessible, e.g., an  
enzyme that releases a component of nutrient value from a more complex  
molecule, perhaps a macromolecule.

## 9. Male Sterility

Male sterility is useful in the production of hybrid seed. It is proposed that male sterility may be produced through expression of novel genes. For example, it has been shown that expression of genes that encode proteins that interfere with development of the male inflorescence and/or gametophyte result in male sterility. Chimeric ribonuclease genes that express in the anthers of transgenic tobacco and oilseed rape have been demonstrated to lead to male sterility (Mariani et al, 1990).

A number of mutations were discovered in maize that confer cytoplasmic male sterility. One mutation in particular, referred to as T cytoplasm, also correlates with sensitivity to Southern corn leaf blight. A DNA sequence, designated TURF-13 (Levings, 1990), was identified that correlates with T cytoplasm. It is proposed that it would be possible through the introduction of TURF-13 via transformation to separate male sterility from disease sensitivity. As it is necessary to be able to restore male fertility for breeding purposes and for grain production it is proposed that genes encoding restoration of male fertility may also be introduced.

## 10. Negative Selectable Markers

Introduction of genes encoding traits that can be selected against may be useful for eliminating undesirable linked genes. It is contemplated that when two or more genes are introduced together by cotransformation that the genes will be linked together on the host chromosome. For example, a gene encoding a *Bt* gene that confers insect resistance on the plant may be introduced into a plant together with a *bar* gene that is useful as a selectable marker and confers resistance to the herbicide Ignite<sup>®</sup> (biazophos) on the plant. However, it may not be desirable to have an insect resistant plant that is also resistant to the herbicide Ignite<sup>®</sup> (biazophos). It is proposed that

one could also introduce an antisense *bar* gene that is expressed in those tissues where one does not want expression of the *bar* gene, e.g., in whole plant parts. Hence, although the *bar* gene is expressed and is useful as a selectable marker, it is not useful to confer herbicide resistance on the whole plant. The *bar* antisense gene is a negative selectable marker.

It is also contemplated that a negative selection is necessary in order to screen a population of transformants for rare homologous recombinants generated through gene targeting. For example, a homologous recombinant may be identified through the inactivation of a gene that was previously expressed in that cell. The antisense gene to neomycin phosphotransferase II (nptII) has been investigated as a negative selectable marker in tobacco (*Nicotiana tabacum*) and *Arabidopsis thaliana* (Xiang, C. and Guerra, D.J. 1993). In this example both sense and antisense npt II genes are introduced into a plant through transformation and the resultant plants are sensitive to the antibiotic kanamycin. An introduced gene that integrates into the host cell chromosome at the site of the antisense nptII gene, and inactivates the antisense gene, will make the plant resistant to kanamycin and other aminoglycoside antibiotics. Therefore, rare site specific recombinants may be identified by screening for antibiotic resistance. Similarly, any gene, native to the plant or introduced through transformation, that when inactivated confers resistance to a compound, may be useful as a negative selectable marker.

It is contemplated that negative selectable markers may also be useful in other ways. One application is to construct transgenic lines in which one could select for transposition to unlinked sites. In the process of tagging it is most common for the transposable element to move to a genetically linked site on the same chromosome. A selectable marker for recovery of rare plants in which transposition has occurred to an unlinked locus would be useful. For example, the enzyme cytosine deaminase may be useful for this purpose (Stouggard, J., 1993). In the presence of this enzyme the compound 5-fluorocytosine is converted to 5-

fluorouracil which is toxic to plant and animal cells. If a transposable element is linked to the gene for the enzyme cytosine deaminase, one may select for transposition to unlinked sites by selecting for transposition events in which the resultant plant is now resistant to 5-fluorocytosine. The parental plants and plants containing transpositions to linked sites will remain sensitive to 5-fluorocytosine. Resistance to 5-fluorocytosine is due to loss of the cytosine deaminase gene through genetic segregation of the transposable element and the cytosine deaminase gene. Other genes that encode proteins that render the plant sensitive to a certain compound will also be useful in this context. For example, T-DNA gene 2 from *Agrobacterium tumefaciens* encodes a protein that catalyzes the conversion of  $\alpha$ -naphthalene acetamide (NAM) to  $\alpha$ -naphthalene acetic acid (NAA) renders plant cells sensitive to high concentrations of NAM (Depicker et al., 1988).

It is also contemplated that negative selectable markers may be useful in the construction of transposon tagging lines. For example, by marking an autonomous transposable element such as Ac, Master Mu, or En/Spn with a negative selectable marker, one could select for transformants in which the autonomous element is not stably integrated into the genome. It is proposed that this would be desirable, for example, when transient expression of the autonomous element is desired to activate in trans the transposition of a defective transposable element, such as Ds, but stable integration of the autonomous element is not desired. The presence of the autonomous element may not be desired in order to stabilize the defective element, i.e., prevent it from further transposing. However, it is proposed that if stable integration of an autonomous transposable element is desired in a plant the presence of a negative selectable marker may make it possible to eliminate the autonomous element during the breeding process.



## **D. Non-Protein-Expressing Sequences**

### **1. RNA-Expressing**

5 DNA may be introduced into corn and other monocots for the purpose of expressing RNA transcripts that function to affect plant phenotype yet are not translated into protein. Two examples are antisense RNA and RNA with ribozyme activity. Both may serve possible functions in reducing or eliminating expression of native or introduced plant genes.

10 Genes may be constructed or isolated, which when transcribed, produce antisense RNA that is complementary to all or part(s) of a targeted messenger RNA(s). The antisense RNA reduces production of the polypeptide product of the messenger RNA. The polypeptide product may be any protein encoded by the  
15 plant genome. The aforementioned genes will be referred to as antisense genes. An antisense gene may thus be introduced into a plant by transformation methods to produce a novel transgenic plant with reduced expression of a selected protein of interest. For example, the protein may be an enzyme that catalyzes a reaction in the plant. Reduction of the enzyme activity may reduce or eliminate products of  
20 the reaction which include any enzymatically synthesized compound in the plant such as fatty acids, amino acids, carbohydrates, nucleic acids and the like. Alternatively, the protein may be a storage protein, such as a zein, or a structural protein, the decreased expression of which may lead to changes in seed amino acid composition or plant morphological changes respectively. The possibilities cited  
25 above are provided only by way of example and do not represent the full range of applications.

30 Genes may also be constructed or isolated, which when transcribed produce RNA enzymes, or ribozymes, which can act as endoribonucleases and catalyze the cleavage of RNA molecules with selected sequences. The cleavage of selected

messenger RNA's can result in the reduced production of their encoded polypeptide products. These genes may be used to prepare novel transgenic plants which possess them. The transgenic plants may possess reduced levels of polypeptides including but not limited to the polypeptides cited above that may be affected by antisense RNA.

It is also possible that genes may be introduced to produce novel transgenic plants which have reduced expression of a native gene product by a mechanism of cosuppression. It has been demonstrated in tobacco, tomato, and petunia (Goring et al, 1991; Smith et al., 1990; Napoli, C. et al., 1990; van der Krol et al., 1990) that expression of the sense transcript of a native gene will reduce or eliminate expression of the native gene in a manner similar to that observed for antisense genes. The introduced gene may encode all or part of the targeted native protein but its translation may not be required for reduction of levels of that native protein.

## **2. Non-RNA-Expressing**

For example, DNA elements including those of transposable elements such as Ds, Ac, or Mu, may be inserted into a gene and cause mutations. These DNA elements may be inserted in order to inactivate (or activate) a gene and thereby "tag" a particular trait. In this instance the transposable element does not cause instability of the tagged mutation, because the utility of the element does not depend on its ability to move in the genome. Once a desired trait is tagged, the introduced DNA sequence may be used to clone the corresponding gene, e.g., using the introduced DNA sequence as a PCR primer together with PCR gene cloning techniques (Shapiro, 1983; Dellaporta et al., 1988). Once identified, the entire gene(s) for the particular trait, including control or regulatory regions where desired may be isolated, cloned and manipulated as desired. The utility of DNA

elements introduced into an organism for purposed of gene tagging is independent of the DNA sequence and does not depend on any biological activity of the DNA sequence, i.e., transcription into RNA or translation into protein. The sole function of the DNA element is to disrupt the DNA sequence of a gene.

5

It is contemplated that unexpressed DNA sequences, including novel synthetic sequences could be introduced into cells as proprietary "labels" of those cells and plants and seeds thereof. It would not be necessary for a label DNA element to disrupt the function of a gene endogenous to the host organism, as the sole function of this DNA would be to identify the origin of the organism. For example, one could introduce a unique DNA sequence into a plant and this DNA element would identify all cells, plants, and progeny of these cells as having arisen from that labelled source. It is proposed that inclusion of label DNAs would enable one to distinguish proprietary germplasm or germplasm derived from such, from unlabelled germplasm.

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Another possible element which may be introduced is a matrix attachment region element (MAR), such as the chicken lysozyme A element (Stief, 1989), which can be positioned around an expressible gene of interest to effect an increase in overall expression of the gene and diminish position dependant effects upon incorporation into the plant genome (Stief *et al.*, 1989; Phi-Van *et al.*, 1990).

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### III. DNA Delivery

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Following the generation of recipient cells, the present invention generally next includes steps directed to introducing an exogenous DNA segment, such as a cDNA or gene, into a recipient cell to create a transformed cell. The frequency of occurrence of cells receiving DNA is believed to be low. Moreover, it is most likely that not all recipient cells receiving DNA segments will result in a transformed cell wherein the DNA is stably integrated into the plant genome and/or expressed.

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Some may show only initial and transient gene expression. However, certain cells from virtually any monocot species may be stably transformed, and these cells developed into transgenic plants, through the application of the techniques disclosed herein.

5

There are many methods for introducing transforming DNA segments into cells, but not all are suitable for delivering DNA to plant cells. Suitable methods are believed to include virtually any method by which DNA can be introduced into a cell, such as by *Agrobacterium* infection, direct delivery of DNA such as, for example, by PEG-mediated transformation of protoplasts (Omirulleh et al., 1993), by desiccation/inhibition-mediated DNA uptake, by electroporation, by agitation with silicon carbide fibers, by acceleration of DNA coated particles, etc. In certain embodiments, acceleration methods are preferred and include, for example, microprojectile bombardment and the like.

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#### **A. Electroporation**

Where one wishes to introduce DNA by means of electroporation, it is contemplated that the method of Krzyzek *et al.* (U.S. Serial Number 07/635,279 filed December 28, 1990, incorporated herein by reference) will be particularly advantageous. In this method, certain cell wall-degrading enzymes, such as pectin-degrading enzymes, are employed to render the target recipient cells more susceptible to transformation by electroporation than untreated cells. Alternatively, recipient cells are made more susceptible to transformation, by mechanical wounding.

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To effect transformation by electroporation one may employ either friable tissues such as a suspension culture of cells, or embryogenic callus, or alternatively, one may transform immature embryos or other organized tissues directly. One would partially degrade the cell walls of the chosen cells by exposing

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them to pectin-degrading enzymes (pectolyases) or mechanically wounding in a controlled manner. Such cells would then be recipient to DNA transfer by electroporation, which may be carried out at this stage, and transformed cells then identified by a suitable selection or screening protocol dependent on the nature of the newly incorporated DNA.

## **B. Microprojectile Bombardment**

A further advantageous method for delivering transforming DNA segments to plant cells is microprojectile bombardment. In this method, particles may be coated with nucleic acids and delivered into cells by a propelling force. Exemplary particles include those comprised of tungsten, gold, platinum, and the like.

It is contemplated that in some instances DNA precipitation onto metal particles would not be necessary for DNA delivery to a recipient cell using microprojectile bombardment. In an illustrative embodiment, non-embryogenic BMS cells were bombarded with intact cells of the bacteria *E. coli* or *Agrobacterium tumefaciens* containing plasmids with either the  $\beta$ -glucoronidase or *bar* gene engineered for expression in maize. Bacteria were inactivated by ethanol dehydration prior to bombardment. A low level of transient expression of the  $\beta$ -glucoronidase gene was observed 24-48 hours following DNA delivery. In addition, stable transformants containing the *bar* gene were recovered following bombardment with either *E. coli* or *Agrobacterium tumefaciens* cells. It is contemplated that particles may contain DNA rather than be coated with DNA. Hence it is proposed that DNA-coated particles may increase the level of DNA delivery via particle bombardment but are not, in and of themselves, necessary.

An advantage of microprojectile bombardment, in addition to it being an effective means of reproducibly stably transforming monocots, is that neither the isolation of protoplasts (Cristou *et al.*, 1988) nor the susceptibility to

*Agrobacterium* infection is required. An illustrative embodiment of a method for delivering DNA into maize cells by acceleration is a Biolistics Particle Delivery System, which can be used to propel particles coated with DNA or cells through a screen, such as a stainless steel or Nytex screen, onto a filter surface covered with corn cells cultured in suspension. The screen disperses the particles so that they are not delivered to the recipient cells in large aggregates. It is believed that a screen intervening between the projectile apparatus and the cells to be bombarded reduces the size of projectiles aggregate and may contribute to a higher frequency of transformation by reducing damage inflicted on the recipient cells by projectiles that are too large.

For the bombardment, cells in suspension are preferably concentrated on filters or solid culture medium. Alternatively, immature embryos or other target cells may be arranged on solid culture medium. The cells to be bombarded are positioned at an appropriate distance below the macroprojectile stopping plate. If desired, one or more screens are also positioned between the acceleration device and the cells to be bombarded. Through the use of techniques set forth herein one may obtain up to 1000 or more foci of cells transiently expressing a marker gene. The number of cells in a focus which express the exogenous gene product 48 hours post-bombardment often range from 1 to 10 and average 1 to 3.

In bombardment transformation, one may optimize the prebombardment culturing conditions and the bombardment parameters to yield the maximum numbers of stable transformants. Both the physical and biological parameters for bombardment are important in this technology. Physical factors are those that involve manipulating the DNA/microprojectile precipitate or those that affect the flight and velocity of either the macro- or microprojectiles. Biological factors include all steps involved in manipulation of cells before and immediately after bombardment, the osmotic adjustment of target cells to help alleviate the trauma associated with bombardment, and also the nature of the transforming DNA, such

as linearized DNA or intact supercoiled plasmids. It is believed that pre-bombardment manipulations are especially important for successful transformation of immature embryos.

5           Accordingly, it is contemplated that one may wish to adjust various of the bombardment parameters in small scale studies to fully optimize the conditions. One may particularly wish to adjust physical parameters such as gap distance, flight distance, tissue distance, and helium pressure. One may also minimize the trauma reduction factors (TRFs) by modifying conditions which influence the  
10           physiological state of the recipient cells and which may therefore influence transformation and integration efficiencies. For example, the osmotic state, tissue hydration and the subculture stage or cell cycle of the recipient cells may be adjusted for optimum transformation. Results from such small scale optimization studies are disclosed herein and the execution of other routine adjustments will be  
15           known to those of skill in the art in light of the present disclosure.

#### **IV.    Production and Characterization of Stable Transgenic Corn**

20           After effecting delivery of exogenous DNA to recipient cells by any of the methods discussed above, the next steps of the invention generally concern identifying the transformed cells for further culturing and plant regeneration. As mentioned above, in order to improve the ability to identify transformants, one may desire to employ a selectable or screenable marker gene as, or in addition to, the  
25           expressible gene of interest. In this case, one would then generally assay the potentially transformed cell population by exposing the cells to a selective agent or agents, or one would screen the cells for the desired marker gene trait.

## A. Selection

An exemplary embodiment of methods for identifying transformed cells involves exposing the bombarded cultures to a selective agent, such as a metabolic inhibitor, an antibiotic, herbicide or the like. Cells which have been transformed and have stably integrated a marker gene conferring resistance to the selective agent used, will grow and divide in culture. Sensitive cells will not be amenable to further culturing.

To use the *bar*-bialaphos or the EPSPS-glyphosate selective system, bombarded tissue is cultured for 0 - 28 days on nonselective medium and subsequently transferred to medium containing from 1-3 mg/l bialaphos or 1-3 mM glyphosate as appropriate. While ranges of 1-3 mg/l bialaphos or 1-3 mM glyphosate will typically be preferred, it is proposed that ranges of 0.1-50 mg/l bialaphos or 0.1-50 mM glyphosate will find utility in the practice of the invention. Tissue can be placed on any porous, inert, solid or semi-solid support for bombardment, including but not limited to filters and solid culture medium. Bialaphos and glyphosate are provided as examples of agents suitable for selection of transformants, but the technique of this invention is not limited to them.

An example of a screenable marker trait is the red pigment produced under the control of the R-locus in maize. This pigment may be detected by culturing cells on a solid support containing nutrient media capable of supporting growth at this stage and selecting cells from colonies (visible aggregates of cells) that are pigmented. These cells may be cultured further, either in suspension or on solid media. The R-locus is useful for selection of transformants from bombarded immature embryos. In a similar fashion, the introduction of the C1 and B genes will result in pigmented cells and/or tissues.

The enzyme luciferase is also useful as a screenable marker in the context of



the present invention. In the presence of the substrate luciferin, cells expressing luciferase emit light which can be detected on photographic or x-ray film, in a luminometer (or liquid scintillation counter), by devices that enhance night vision, or by a highly light sensitive video camera, such as a photon counting camera. All of these assays are nondestructive and transformed cells may be cultured further following identification. The photon counting camera is especially valuable as it allows one to identify specific cells or groups of cells which are expressing luciferase and manipulate those in real time.

It is further contemplated that combinations of screenable and selectable markers will be useful for identification of transformed cells. In some cell or tissue types a selection agent, such as bialaphos or glyphosate, may either not provide enough killing activity to clearly recognize transformed cells or may cause substantial nonselective inhibition of transformants and nontransformants alike, thus causing the selection technique to not be effective. It is proposed that selection with a growth inhibiting compound, such as bialaphos or glyphosate at concentrations below those that cause 100% inhibition followed by screening of growing tissue for expression of a screenable marker gene such as luciferase would allow one to recover transformants from cell or tissue types that are not amenable to selection alone. In an illustrative embodiment embryogenic type II callus of *Zea mays* L. was selected with sub-lethal levels of bialaphos. Slowly growing tissue was subsequently screened for expression of the luciferase gene and transformants were identified. In this example, neither selection nor screening conditions employed were sufficient in and of themselves to identify transformants. Therefore it is proposed that combinations of selection and screening will enable one to identify transformants in a wider variety of cell and tissue types.

## B. Regeneration and Seed Production

Cells that survive the exposure to the selective agent, or cells that have been scored positive in a screening assay, may be cultured in media that supports  
5 regeneration of plants. In an exemplary embodiment, the inventors have modified MS and N6 media (see Table 1) by including further substances such as growth regulators. A preferred growth regulator for such purposes is dicamba or 2,4-D. However, other growth regulators may be employed, including NAA, NAA + 2,4-D or perhaps even picloram. Media improvement in these and like ways was found  
10 to facilitate the growth of cells at specific developmental stages. Tissue is preferably maintained on a basic media with growth regulators until sufficient tissue is available to begin plant regeneration efforts, or following repeated rounds of manual selection, until the morphology of the tissue is suitable for regeneration, at least two weeks, then transferred to media conducive to maturation of  
15 embryoids. Cultures are transferred every two weeks on this medium. Shoot development will signal the time to transfer to medium lacking growth regulators.

The transformed cells, identified by selection or screening and cultured in an appropriate medium that supports regeneration, will then be allowed to mature into  
20 plants. Developing plantlets are transferred to soilless plant growth mix, and hardened, e.g., in an environmentally controlled chamber at about 85% relative humidity, 600 ppm CO<sub>2</sub>, and 25-250 microeinsteins m<sup>-2</sup>·s<sup>-1</sup> of light. Plants are preferably matured either in a growth chamber or greenhouse. Plants are regenerated from about 6 weeks to 10 months after a transformant is identified,  
25 depending on the initial tissue. During regeneration, cells are grown on solid media in tissue culture vessels. Illustrative embodiments of such vessels are petri dishes and Plant Con®s.. Regenerating plants are preferably grown at about 19 to 28°C. After the regenerating plants have reached the stage of shoot and root development, they may be transferred to a greenhouse for further growth and  
30 testing.

In one study,  $R_0$  plants were regenerated from transformants of an A188 x B73 suspension culture line (SC82), and these plants exhibited a phenotype expected of the genotype of hybrid A188 X B73 from which the callus and culture were derived. The plants were similar in height to seed-derived A188 plants (3-5 ft tall) but had B73 traits such as anthocyanin accumulation in stalks and prop roots, and the presence of upright leaves. It would also be expected that some traits in the transformed plants would differ from their source, and indeed some variation will likely occur.

In an exemplary embodiment, the proportion of regenerating plants derived from transformed callus that successfully grew and reached maturity after transfer to the greenhouse was 97% (73 of 76).  $R_0$  plants in the greenhouse are tested for fertility by backcrossing the transformed plants with seed-derived plants by pollinating the  $R_0$  ears with pollen from seed derived inbred plants and this resulted in kernel development. In addition, pollen was collected from  $R_0$  plants and used to pollinate seed derived inbred plants, resulting in kernel development. Although fertility can vary from plant to plant greater than 100 viable progeny can be routinely recovered from each transformed plant through use of both the ear and pollen for doing crosses.

Note, however, that occasionally kernels on transformed plants may require embryo rescue due to cessation of kernel development and premature senescence of plants. To rescue developing embryos, they are excised from surface-disinfected kernels 10-20 days post-pollination and cultured. An embodiment of media used for culture at this stage comprises MS salts, 2% sucrose, and 5.5 g/l agarose. In an illustrative embodiment of embryo rescue, large embryos (defined as greater than 3 mm in length) are germinated directly on an appropriate media. Embryos smaller than that were cultured for one week on media containing the above ingredients along with  $10^{-5}$ M abscisic acid and then transferred to growth regulator-free medium for germination.

Progeny may be recovered from the transformed plants and tested for expression of the exogenous expressible gene by localized application of an appropriate substrate to plant parts such as leaves. In the case of *bar* transformed plants, it was found that transformed parental plants ( $R_0$ ) and their progeny ( $R_1$ ) exhibited no bialaphos-related necrosis after localized application of the herbicide Basta® to leaves, if there was functional PAT activity in the plants as assessed by an *in vitro* enzymatic assay. In one study, of 28 progeny ( $R_1$ ) plants tested, 50% (N=14) had PAT activity. All PAT positive progeny tested contained *bar*, confirming that the presence of the enzyme and the resistance to bialaphos were associated with the transmission through the germline of the marker gene.

### **C. Characterization**

To confirm the presence of the exogenous DNA or "transgene (s)" in the regenerating plants, a variety of assays may be performed. Such assays include, for example, "molecular biological" assays, such as Southern and Northern blotting and PCR; "biochemical" assays, such as detecting the presence of a protein product, e.g., by immunological means (ELISAs and Western blots) or by enzymatic function; plant part assays, such as leaf or root assays; and also, by analyzing the phenotype of the whole regenerated plant.

#### **1. DNA Integration, RNA Expression and Inheritance**

Genomic DNA may be isolated from callus cell lines or any plant parts to determine the presence of the exogenous gene through the use of techniques well known to those skilled in the art. Note, that intact sequences will not always be present, presumably due to rearrangement or deletion of sequences in the cell.

The presence of DNA elements introduced through the methods of this invention may be determined by polymerase chain reaction (PCR). Using this

technique discreet fragments of DNA are amplified and detected by gel electrophoresis. This type of analysis permits one to determine whether a gene is present in a stable transformant, but does not prove integration of the introduced gene into the host cell genome. It is the experience of the inventors, however, that DNA has been integrated into the genome of all transformants that demonstrate the presence of the gene through PCR analysis. In addition, it is not possible using PCR techniques to determine whether transformants have exogenous genes introduced into different sites in the genome, i.e., whether transformants are of independent origin. It is contemplated that using PCR techniques it would be possible to clone fragments of the host genomic DNA adjacent to an introduced gene.

Positive proof of DNA integration into the host genome and the independent identities of transformants may be determined using the technique of Southern hybridization. Using this technique specific DNA sequences that were introduced into the host genome and flanking host DNA sequences can be identified. Hence the Southern hybridization pattern of a given transformant serves as an identifying characteristic of that transformant. In addition it is possible through Southern hybridization to demonstrate the presence of introduced genes in high molecular weight DNA, i.e., confirm that the introduced gene has been integrated into the host cell genome. The technique of Southern hybridization provides information that is obtained using PCR e.g., the presence of a gene, but also demonstrates integration into the genome and characterizes each individual transformant.

It is contemplated that using the techniques of dot or slot blot hybridization which are modifications of Southern hybridization techniques one could obtain the same information that is derived from PCR, e.g., the presence of a gene.

Both PCR and Southern hybridization techniques can be used to demonstrate transmission of a transgene to progeny. It is the experience of the inventors that

in most instances the characteristic Southern hybridization pattern for a given transformant will segregate in progeny as one or more Mendelian genes (Spencer et al., 1992; Spencer et al, in press) indicating stable inheritance of the transgene. For example, in one study, of 28 progeny ( $R_1$ ) plants tested, 50% ( $N = 14$ ) contained *bar*, confirming transmission through the germline of the marker gene. The nonchimeric nature of the callus and the parental transformants ( $R_0$ ) was suggested by germline transmission and the identical Southern blot hybridization patterns and intensities of the transforming DNA in callus,  $R_0$  plants and  $R_1$  progeny that segregated for the transformed gene.

Whereas DNA analysis techniques may be conducted using DNA isolated from any part of a plant, RNA will only be expressed in particular cells or tissue types and hence it will be necessary to prepare RNA for analysis from these tissues. PCR techniques may also be used for detection and quantitation of RNA produced from introduced genes. In this application of PCR it is first necessary to reverse transcribe RNA into DNA, using enzymes such as reverse transcriptase, and then through the use of conventional PCR techniques amplify the DNA. In most instances PCR techniques, while useful, will not demonstrate integrity of the RNA product. Further information about the nature of the RNA product may be obtained by Northern blotting. This technique will demonstrate the presence of an RNA species and give information about the integrity of that RNA. The presence or absence of an RNA species can also be determined using dot or slot blot Northern hybridizations. These techniques are modifications of Northern blotting and will only demonstrate the presence or absence of an RNA species.

## 2. Gene Expression

While Southern blotting and PCR may be used to detect the gene(s) in question, they do not provide information as to whether the gene is being expressed. Expression may be evaluated by specifically identifying the protein products of the introduced genes or evaluating the phenotypic changes brought about by their expression.

Assays for the production and identification of specific proteins may make use of physical-chemical, structural, functional, or other properties of the proteins. Unique physical-chemical or structural properties allow the proteins to be separated and identified by electrophoretic procedures, such as native or denaturing gel electrophoresis or isoelectric focussing, or by chromatographic techniques such as ion exchange or gel exclusion chromatography. The unique structures of individual proteins offer opportunities for use of specific antibodies to detect their presence in formats such as an ELISA assay. Combinations of approaches may be employed with even greater specificity such as western blotting in which antibodies are used to locate individual gene products that have been separated by electrophoretic techniques. Additional techniques may be employed to absolutely confirm the identity of the product of interest such as evaluation by amino acid sequencing following purification. Although these are among the most commonly employed, other procedures may be additionally used.

Assay procedures may also be used to identify the expression of proteins by their functionality, especially the ability of enzymes to catalyze specific chemical reactions involving specific substrates and products. These reactions may be followed by providing and quantifying the loss of substrates or the generation of products of the reactions by physical or chemical procedures. Examples are as varied as the enzyme to be analyzed and may include assays for PAT enzymatic activity by following production of radiolabeled acetylated phosphinothricin from

phosphinothricin and  $^{14}\text{C}$ -acetyl CoA or for anthranilate synthase activity by following loss of fluorescence of anthranilate, to name two.

Very frequently the expression of a gene product is determined by evaluating the phenotypic results of its expression. These assays also may take many forms including but not limited to analyzing changes in the chemical composition, morphology, or physiological properties of the plant. Chemical composition may be altered by expression of genes encoding enzymes or storage proteins which change amino acid composition and may be detected by amino acid analysis, or by enzymes which change starch quantity which may be analyzed by near infrared reflectance spectrometry. Morphological changes may include greater stature or thicker stalks. Most often changes in response of plants or plant parts to imposed treatments are evaluated under carefully controlled conditions termed bioassays. An example is to evaluate resistance to insect feeding.

\* \* \*

The inventors have been successful in producing fertile transgenic monocot plants (maize) where others have failed. Aspects of the methods of the present invention for producing the fertile, transgenic corn plants comprise, but are not limited to, isolation of recipient cells using media conducive to specific growth patterns, choice of selective systems that permit efficient detection of transformation; modifications of DNA delivery methods to introduce genetic vectors with exogenous DNA into cells; invention of methods to regenerate plants from transformed cells at a high frequency; and the production of fertile transgenic plants capable of surviving and reproducing.



## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Schematic representation of plasmids (vectors) used in bombardment experiments.

FIG. 1 (A). Schematic representation of the expression cassette of pDPG165 containing the *bar* gene.

FIG. 1 (B). Schematic representation of the expression cassette of pDPG208 containing the *uidA* gene encoding  $\beta$ -glucoronidase (GUS).

FIG. 1 (C). Map of plasmid pDPG165 containing the *bar* gene.

FIG. 1 (D) Map of plasmid pDPG208 containing the *uidA* gene.

FIG. 1 (E) Map of plasmid pAGUS1, also known as pDPG141, in which the 5'-noncoding and 5'-coding sequences were modified to incorporate the Kozak consensus sequence and HindIII restriction site. The nucleotide sequence is ~~seq id~~ *seq id no:2*

~~no:2.~~

FIG 1 (F). Restriction map of the plasmid pDPG237 containing the Sn:bol3 cDNA.

FIG. 1 (G). Map of plasmid pDPG232 incorporating the Rsn cDNA with a 35S promoter and Tr 7 3' end.

FIG. 1 (H). Map of plasmid pDPG313 containing the *aroA* gene and the 35S-histone fusion promoter in addition to the *bar* expression cassette.

FIG. 1 (I). Map of plasmid pDPG314 containing the *aroA* gene and the 35S-histone

fusion promoter in addition to the *bar* expression cassette.

FIG. 1 (J). Map of plasmid pDPG315 containing the *aroA* gene and the histone fusion promoter in addition to the *bar* expression cassette.

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FIG. 1 (K). Map of plasmid pDPG316 containing the *aroA* gene and the histone fusion promoter in addition to the *bar* expression cassette.

FIG. 1 (L). Map of plasmid pDPG317 containing the *aroA* gene and the 35S-histone fusion promoter in addition to the *bar* expression cassette.

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FIG. 1 (M). Map of plasmid pDPG318 containing the *aroA* gene and the  $\alpha$ -tubulin promoter in addition to the *bar* expression cassette.

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FIG. 1 (N). Map of plasmid pDPG319 containing the *aroA* gene and the  $\alpha$ -tubulin promoter in addition to the *bar* expression cassette.

FIG. 1 (O). Map of plasmid pDPG290 containing the *B. thuringiensis* crystal toxin protein gene *lab6* with a 35S promoter.

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FIG. 1 (P). Map of plasmid pDPG300 containing the *B. thuringiensis* crystal toxin protein gene *lab6* with a 35S promoter in addition to the *bar* expression cassette from pDPG165.

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FIG. 1 (Q). Map of plasmid pDPG301 containing the *B. thuringiensis* crystal toxin protein gene *lab6* with a 35S promoter in addition to the *bar* expression cassette from pDPG165.

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FIG. 1 (R). Map of plasmid pDPG302 containing the *B. thuringiensis* crystal toxin protein gene *lab6* with a 35S promoter in addition to the *bar* expression

cassette from pDPG165.

FIG. 1 (S). Map of plasmid pDPG303 containing the *B. thuringiensis* crystal toxin protein gene *lab6* with a 35S promoter in addition to the *bar* expression cassette from pDPG165.

FIG. 1 (T). Map of plasmid pDPG386, a plasmid containing the wheat dwarf virus replicon and containing a neomycin phosphotransferase II gene. This virus replicates in plant cells as well as bacteria.

FIG. 1 (U). Map of plasmid pDPG387, a plasmid containing the wheat dwarf virus replicon and containing a neomycin phosphotransferase II gene and the *uidA* gene encoding GUS. This virus replicates in plant cells as well as bacteria.

FIG. 1 (V). Map of plasmid pDPG388, a plasmid containing the wheat dwarf virus replicon and containing a neomycin phosphotransferase II gene and the *bar*. This virus replicates in plant cells as well as bacteria.

FIG. 1 (W). Map of plasmid pDPG389, a plasmid containing the wheat dwarf virus replicon and containing a neomycin phosphotransferase II gene and the *bar* gene. This virus replicates in plant cells as well as bacteria.

FIG. 1 (X). Map of plasmid pDPG140 .

FIG. 1 (Y). Map of plasmid pDPG172 containing the luciferase gene and the maize alcohol dehydrogenase I promoter and intron one.

FIG. 1 (Z). Map of plasmid pDPG425 containing a maize EPSPS gene mutated to confer resistance to glyphosate.

FIG. 1 (AA). Map of plasmid pDPG427 containing a maize EPSPS gene mutated to confer resistance to glyphosate.

FIG. 1 (BB). Map of plasmid pDPG451 containing the 35S promoter - adh intron- *mtlD*- Tr7 expression cassette. Expression of this cassette will lead to accumulation of mannitol in the cells.

FIG. 1 (CC). Map of plasmid pDPG354 containing a synthetic *Bt* gene (see figure 12).

FIG. 1 (DD). Map of plasmid pDPG344 containing the proteinase inhibitor II gene from tomato.

FIG. 1 (EE). Map of plasmid pDPG337 containing a synthetic *Bt* gene (see figure 12).

FIG. 2. Appearance of cell colonies which emerge on selection plates with bialaphos. Such colonies appear 6-7 weeks after bombardment.

FIG. 2 (A) SC82 bialaphos-resistant colony selected on 1 mg/l bialaphos.

FIG. 2 (B) Embryogenic SC82 bialaphos-resistant callus selected and maintained on 1 mg/l bialaphos.

FIG. 3. Phosphinothricin acetyl transferase (PAT) activity in embryogenic SC82 callus transformants designated E1-E11 and a nonselected control (EO). 25  $\mu$ g of protein extract were loaded per lane. B13 is a BMS-*bar* transformant. BMS is Black Mexican Sweet corn. Activities of the different transformants varied approximately 10 fold based on the intensities of the bands.

FIG. 4. Integration of the bar gene in bialaphos-resistant SC82 callus isolates E1-E11. DNA gel blot of genomic DNA (4  $\mu$ g/digest) from E1-E11 and a nonselected control (EO) digested with EcoRI and HindIII. The molecular weights in kb are shown on the left and right. The blot was hybridized with  $^{32}$ P-labeled *bar* from pDPG165 ( $\sim 25 \times 10^6$  Cerenkov cpm). Lanes designated 1 and 5 copies refer to the diploid genome and contain 1.9 and 9.5 pg respectively of the 1.9 kb *bar* expression unit released from pDPG165 with EcoRI and HindIII.

FIG. 5. Integration of exogenous genes in bialaphos-resistant SC716 isolates R1-R21.

FIG. 5 (A) DNA gel blot of genomic DNA (6  $\mu$ g/digest) from transformants isolated from suspension culture of A188 x B73 (SC716), designated R1-R21, were digested with EcoRI and HindIII and hybridized to  $^{32}$ P-labeled *bar* probe ( $\sim 10 \times 10^6$  Cerenkov cpm). Molecular weight markers in kb are shown on the left and right. Two copies of the *bar* expression unit per diploid genome is 5.7 pg of the 1.9 kb EcoRI/Hind fragment from pDPG165.

FIG. 5 (B) The blot from A was washed and hybridized with  $^{32}$ P-labelled GUS probe ( $\sim 35 \times 10^6$  Cerenkov cpm). Two copies of the 2.1 kb GUS-containing EcoRI/HindIII fragment from pDPG208 is 6.3 pg.

FIG. 6. Histochemical determination of GUS activity in bar-transformed SC82 callus line Y13. This bialaphos-resistant callus line, Y13, which contained intact GUS coding sequences was tested for GUS activity three months post-bombardment. In this figure, differential staining of the callus was observed.

FIG. 7. Mature R<sub>0</sub> Plant, Developing Kernels and Progeny.

FIG. 7 (A). Mature transgenic R<sub>0</sub> plant regenerated from an E2/E5 callus.

FIG. 7 (B) Progeny derived from an E2/E5 plant by embryo rescue; segregant bearing the resistance gene on the right, and lacking the gene on the left.

5 FIG. 7 (C) Using pollen from transformed  $R_1$  plants to pollinate B73 ears, large numbers of seed have been recovered.

FIG. 7 (D) A transformed ear from an  $R_1$  plant crossed with pollen from a non-transformed inbred plant.

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FIG. 8. Functional Expression of Introduced Genes in Transformed  $R_0$  and  $R_1$  Plants.

15 FIG. 8(A) Basta<sup>R</sup> resistance in transformed  $R_0$  plants. A Basta<sup>R</sup> solution was applied to a large area (about 4 x 8 cm) in the center of leaves of nontransformed A188 x B73 plant (left) and a transgenic  $R_0$  E3/E4/E6 plant (right).

20 FIG. 8 (B) Basta<sup>R</sup> resistance in transformed  $R_1$  plants. Basta<sup>R</sup> was also applied to leaves of four  $R_1$  plants; two plants without *bar* (left) and two plants containing *bar* (right). The herbicide was applied to  $R_1$  plants in 1 cm circles to four locations on each leaf, two on each side of the midrib. Photographs were taken six days after application.

25 FIG. 8 (C) GUS activity in leaf tissue of a transgenic  $R_0$  plant. Histochemical determination of GUS activity in leaf tissue of a plant regenerated from cotransformed callus line Y13 (right) and a nontransformed tissue culture derived plant (left). Bar = 1 cm.

30 FIG. 8 (D) Light micrograph of the leaf segment from a Y13 plant shown in

(C), observed in surface view under bright field optics. GUS activity was observed in many cell types throughout the leaf tissue (magnification = 230X). (E) Light micrograph as in (D) of control leaf.

FIG. 9. PAT Activity in Protein Extracts of R<sub>0</sub> Plants. Extracts from one plant derived from each of the four transformed regenerable callus lines from a suspension culture of A188 x B73, SC82 (E10, E11, E2/E5, and E3/E4/E6) were tested for PAT activity (The designations E2/E5 and E3/E4/E6 represent transformed cell lines with identical DNA gel blot hybridization patterns; the isolates were most likely separated during the culturing and selection process.) Protein extracts from a nontransformed B73 plant and a Black Mexican Sweet (BMS) cell culture *bar* transformant were included as controls. Approximately 50 micrograms of total protein was used per reaction.

FIG. 10. DNA Gel Blot Analysis of Genomic DNA from Transformed Callus and Corresponding R<sub>0</sub> Plants Probed with *bar*. Genomic DNA was digested with EcoRI and HindIII, which released the 1.9 kb *bar* expression unit (CaMV 35S promoter-*bar*-Tr7 3'-end) from pDPG165, the plasmid used for microprojectile bombardment transformation of SC82 cells, and hybridized to *bar*. The molecular weights in kb are shown on the left and right. Lanes designated E3/E4/E6, E11, E2/E5, and E10 contained 5 µg of either callus (C) or R<sub>0</sub> plant DNA. The control lane contained DNA from a nontransformed A188 X B73 plant. The lane designated "1 copy" contained 2.3 pg of the 1.9 kb EcoRI/HindIII fragment from pDPG165 representing one copy per diploid genome.

FIG. 11. PAT Activity and DNA Gel Blot Analysis of Segregating Progeny of E2/E5 R<sub>0</sub> Plants.

FIG. 11 (A) Analysis of PAT activity in ten progeny (lanes a-j) and a nontransformed control plant (lane k). Lanes designated a, b-h, i, and j contained

protein extracts from progeny of separate parental R<sub>0</sub> plants. The lane designated callus contained protein extract from E2/E5 callus. Approximately 25 micrograms of total protein were used per reaction.

5            FIG. 11 (B) DNA gel blot analysis of genomic DNA isolated from the ten progeny analyzed in A. Genomic DNA (5 µg/lane) was digested with SmaI, which releases a 0.6 kb fragment containing *bar* from pDPG165, and hybridized with *bar* probe. The lane designated R<sub>0</sub> contained DNA from the R<sub>0</sub> parent of progeny a. The lane designated 1 copy contained pDPG165 digested with SmaI to represent  
10            approximately 1 copy of the 0.6 kb fragment per diploid genome (0.8 pg).

              FIG. 12        DNA sequence of a synthetic Bt gene coding for the toxin portion of the endotoxin protein produced by *Bacillus thuringiensis* subsp.kurstaki strain HD73 (M.J. Adang et al. 1985 ). This gene was synthesized and assembled  
15            using standard techniques to contain codons that are more preferred for translation in maize cells. A translation stop codon was introduced after the 613th codon to terminate the translation and allow synthesis of a Bt endotoxin protein consisting of the first 613 amino acids (including the f-met) of the Bt protein. The nucleic acid sequence is represented by seq id no:10 and the amino acid sequence by seq id  
20            no:11.

              FIG. 13        DNA sequence of a synthetic Bt gene coding for the toxin portion of the endotoxin protein produced by *Bacillus thuringiensis* strain HD1. This gene was synthesized and assembled using standard techniques to contain  
25            codons that are more preferred for translation in maize cells. The nucleic acid sequence is represented by ~~seq id no:12~~ and the amino acid sequence by ~~seq id no:13~~.  
B            ~~no:13~~.



## DESCRIPTION OF THE PREFERRED EMBODIMENTS

For the first time, fertile transgenic maize plants have been produced, opening the door to new vistas of crop improvement based on *in vitro* genetic transformation. The inventors have succeeded where others have failed by combining and modifying numerous steps in the overall process leading from somatic cell to transgenic plant. Although the methods disclosed herein are part of a unified process, for illustrative purposes they may be subdivided into: culturing cells to be recipients for exogenous DNA; cryopreserving recipient cells; constructing vectors to deliver the DNA to cells; delivering DNA to cells; assaying for successful transformations; using selective agents if necessary to isolate stable transformants; regenerating plants from transformants; assaying those plants for gene expression and for identification of the exogenous DNA sequences; determining whether the transgenic plants are fertile; and producing offspring of the transgenic plants. The invention also relates to transformed maize cells, transgenic plants and pollen produced by said plants.

### **I. Recipient Cells**

Tissue culture requires media and controlled environments. "Media" refers to the numerous nutrient mixtures that are used to grow cells *in vitro*, that is, outside of the intact living organism. The medium is usually a suspension of various categories of ingredients (salts, amino acids, growth regulators, sugars, buffers) that are required for growth of most cell types. However, each specific cell type requires a specific range of ingredient proportions for growth, and an even more specific range of formulas for optimum growth. Rate of cell growth will also vary among cultures initiated with the array of media that permit growth of that cell type.

Nutrient media is prepared as a liquid, but this may be solidified by adding

the liquid to materials capable of providing a solid support. Agar is most commonly used for this purpose. Bactoagar, Hazelton agar, Gelrite, and Gelgro are specific types of solid support that are suitable for growth of plant cells in tissue culture.

5           Some cell types will grow and divide either in liquid suspension or on solid media. As disclosed herein, maize cells will grow in suspension or on solid medium, but regeneration of plants from suspension cultures requires transfer from liquid to solid media at some point in development. The type and extent of differentiation of cells in culture will be affected not only by the type of media used  
10           and by the environment, for example, pH, but also by whether media is solid or liquid. Table 1 illustrates the composition of various media useful for creation of recipient cells and for plant regeneration.

**B.     Culturing Cells to**  
15           **be Recipients for Transformation**

          It is believed by the inventors that the ability to prepare and cryopreserve cultures of maize cells is important to certain aspects of the present invention, in that it provides a means for reproducibly and successfully preparing cells for  
20           particle-mediated transformation, electroporation, or other methods of DNA introduction. The studies described below set forth techniques which have been successfully applied by the inventors to generate transformable and regenerable cultures of maize cells. A variety of different types of media have been developed by the inventors and employed in carrying out various aspects of the invention.  
25           The following table, Table 1, sets forth the composition of the media preferred by the inventors for carrying out these aspects of the invention.

**Table 1: Illustrative Embodiments of Tissue Culture Media Which are Used for Type II Callus Development, Development of Suspension Cultures and Regeneration of Plant Cells (Specifically Maize Cells)**

<b>MEDIA NO.</b>	<b>BASAL MEDIUM</b>	<b>SUCROSE</b>	<b>pH</b>	<b>OTHER COMPONENTS** (Amount/L)</b>
7	MS*	2%	6.0	.25mg thiamine .5mg BAP .5mg NAA Bactoagar
10	MS	2%	6.0	.25mg thiamine 1mg BAP 1mg 2,4-D 400 mg L-proline Bactoagar
19	MS	2%	6.0	.25mg thiamine .25mg BAP .25mg NAA Bactoagar
20	MS	3%	6.0	.25mg 1mg BAP 1mg NAA Bactoagar
52	MS	2%	6.0	.25mg thiamine 1mg 2,4-D 10 <sup>-7</sup> M ABA BACTOAGAR
101	MS	3%	6.0	MS vitamins 100mg myo-inositol Bactoagar

<b>MEDIA NO.</b>	<b>BASAL MEDIUM</b>	<b>SUCROSE</b>	<b>pH</b>	<b>OTHER COMPONENTS** (Amount/L)</b>
142	MS	6%	6.0	MS vitamins 5 mg BAP 0.186mg NAA 0.175mg IAA 0.403mg 2IP Bactoagar
157	MS	6%	6.0	MS vitamins 100mg myo-inositol Bactoagar
163	MS	3%	6.0	MS vitamins 3.3mg dicamba 100 mg myo-inositol Bactoagar
171	MS	3%	6.0	MS vitamins .25mg 2,4-D 10mg BAP 100 mg myo-inositol Bactoagar
173	MS	6%	6.0	MS vitamins 5 mg BAP .186mg NAA .175mg IAA .403mg 2IP $10^{-7}$ M ABA 200mg myo-inositol Bactoagar
177	MS	3%	6.0	MS vitamins .25 mg 2,4-D 10mg BAP $10^{-7}$ M ABA 100mg myo-inositol Bactoagar

<b>MEDIA NO.</b>	<b>BASAL MEDIUM</b>	<b>SUCROSE</b>	<b>pH</b>	<b>OTHER COMPONENTS** (Amount/L)</b>
185	MS	-	5.8	3mg BAP .04mg NAA RT vitamins 1.65mg thiamine 1.38g L-proline 20g sorbitol Bactoagar
189	MS	-	5.8	3mg BAP .04mg NAA .5mg niacin 800mg L-asparagine 100mg casaminio acids 20g sorbitol 1.4g L-proline 100mg myo-inositol Gelgro
201	N6	2%	5.8	N6 vitamins 2mg L-glycine 1mg 2,4-D 100mg casein hydrolysate 2.9g L-proline Gelgro
205	N6	2%	5.8	N6 vitamins 2mg L-glycine .5mg 2,4-D 100mg casein hydrolysate 2.9g L-proline Gelgro
209	N6	6%	5.8	N6 vitamins 2mg L-glycine 100mg casein hydrolysate 0.69g L-proline Bactoagar

<b>MEDIA NO.</b>	<b>BASAL MEDIUM</b>	<b>SUCROSE</b>	<b>pH</b>	<b>OTHER COMPONENTS** (Amount/L)</b>
210	N6	3%	5.5	N6 vitamins 2mg 2,4-D 250mg Ca pantothenate 100mg myo-inositol 790 mg L-asparagine 100 mg casein hydrolypate 1.4g L-proline Hazelton agar**** 2mg L-glycine
212	N6	3%	5.5	N6 vitamins 2mg L-glycine 2mg 2,4-D 250mg Ca pantothenate 100mg myo-inositol 100mg casein hydrolysate 1.4g L-proline Hazelton agar****
227	N6	2%	5.8	N6 vitamins 2mg L-glycine 13.2mg dicamba 100mg casein hydrolysate 2.9g L-proline Gelgro
273	N6	2%	5.8	N6 vitamins 2mg L-glycine 1mg 2,4-D 16.9mg AgNO <sub>3</sub> 100mg casein hydrolysate 2.9g L-proline
279	N6	2%	5.8	3.3mg dicamba 1mg thiamine .5mg niacin 800mg L-asparagine 100mg casein hydrolysate 100mg myoinositol 1.4g L-proline Gelgro****

MEDIA NO.	BASAL MEDIUM	SUCROSE	pH	OTHER COMPONENTS** (Amount/L)
288	N6	3%		3.3mg dicamba 1mg thiamine .5mg niacin .8g L-asparagine 100mg myo-inositol 1.4g L-proline 100mg casein hydrolysate 16.9mg AgNO <sub>3</sub> Gelgro
401	MS	3%	6.0	3.73mg Na <sub>2</sub> EDTA .25mg thiamine 1mg 2,4-D 2mg NAA 200mg casein hydrolysate 500mg K <sub>2</sub> SO <sub>4</sub> 400mg KH <sub>2</sub> PO <sub>4</sub> 100mg myo-inositol
402	MS	3%	6.0	3.73mg Na <sub>2</sub> EDTA .25mg thiamine 1mg 2,4-D 200mg casein hydrolysate 2.9g L-proline 500mg K <sub>2</sub> SO <sub>4</sub> 400mg KH <sub>2</sub> PO <sub>4</sub> 100mg myo-inositol
409	MS	3%	6.0	3.73mg Na <sub>2</sub> EDTA .25mg thiamine 9.9mg dicamba 200mg casein hydrolysate 2.9g L-proline 500mg K <sub>2</sub> SO <sub>4</sub> 400mg KH <sub>2</sub> PO <sub>4</sub> 100mg myo-inositol
401	Clark's Medium***	2%	5.7	

<b>MEDIA NO.</b>	<b>BASAL MEDIUM</b>	<b>SUCROSE</b>	<b>pH</b>	<b>OTHER COMPONENTS** (Amount/L)</b>
607	1/2 x MS	3%	5.8	1mg thiamine 1mg niacin Gelrite
615	MS	3%	6.0	MS vitamins 6mg BAP 100mg myo-inositol Bactoagar
617	1/2 x MS	1.5%	6.0	MS vitamins 50mg myo-inositol Bactoagar
708	N6	2%	5.8	N6 vitamins 2mg L-glycine 1.5mg 2,4-D 200mg casein hydrolysate 0.69g L-proline Gelrite
721	N6	2%	5.8	3.3mg dicamba 1mg thiamine .5mg niacin 800mg L-asparagine 100mg myo-inositol 100mg casein hydrolysate 1.4g L-proline 54.65g mannitol Gelgro
726	N6	3%	5.8	3.3mg dicamba .5 mg niacin 1mg thiamine 800mg L-asparagine 100mg myo-inositol 100mg casein hydrolysate 1.4g L-proline



<b>MEDIA NO.</b>	<b>BASAL MEDIUM</b>	<b>SUCROSE</b>	<b>pH</b>	<b>OTHER COMPONENTS** (Amount/L)</b>
727	N6	3%	5.8	N6 vitamins 2mg L-glycine 9.9mg dicamba 100mg casein hydrolysate 2.9g L-proline Gelgro
728	N6	3%	5.8	N6 vitamins 2mg L-glycine 9.9mg dicamba 16.9mg AgNO <sub>3</sub> 100mg casein hydrolysate 2.9g L-proline Gelgro
734	N6	2%	5.8	N6 vitamins 2mg L-glycine 1.5mg 2,4-D 14g Fe sequestreene (replaces Fe-EDTA) 200mg casein hydrolysate 0.69g L-proline Gelrite
735	N6	2%	5.8	1mg 2,4-D .5mg niacin .91g L-asparagine 100mg myo-inositol 1mg thiamine .5g MES .75g MgCl <sub>2</sub> 100mg casein hydrolysate 0.69g L-proline Gelgro

MEDIA NO.	BASAL MEDIUM	SUCROSE	pH	OTHER COMPONENTS** (Amount/L)
2004	N6	3%	5.8	1 mg thiamine 0.5 mg niacin 3.3 mg dicamba 17 mg AgNO <sub>3</sub> 1.4 g L-proline 0.8 g L-asparagine 100 mg casein hydrolysate 100 mg myo-inositol Gelrite
2008	N6	3%	5.8	1 mg thiamine 0.5 mg niacin 3.3 mg dicamba 1.4 g L-proline 0.8 g L-asparagine Gelrite

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10 \* Basic MS medium described in Murashige and Skoog (1962). This medium is typically modified by decreasing the NH<sub>4</sub>NO<sub>3</sub> from 1.64 g/l to 1.55 g/l, and omitting the pyridoxine HCl, nicotinic acid, myo-inositol and glycine.

15 \*\* NAA = Naphthol Acetic Acid  
IAA = Indole Acetic Acid  
2-IP = 2, isopentyl adenine  
2,4-D = 2, 4-Dichlorophenoxyacetic Acid  
BAP = 6-benzyl aminopurine  
ABA = abscisic acid

20 \*\*\* Basic medium described in Clark (1982)

\*\*\*\* These media may be made with or without solidifying agent.

A number of transformable maize cultures have been developed using the protocols

outlined in the following examples. A compilation of the cultures initiated and tested for transformability is set forth in Table 2, with the results of the studies given in the two right-hand columns. The Table indicates the general selection protocol that was used for each of these cultures. The numeral designations under "Protocol" represent the following:

1. Tissue (suspension) was plated on filters, bombarded and then filters were transferred to culture medium. After 2-7 days, the filters were transferred to selective medium. Approximately 3 weeks after bombardment, tissue was picked from filters as separate callus clumps onto fresh selective medium.
2. As in 1. above, except after bombardment the suspension was put back into liquid - subjected to liquid selection for 7-14 days and then pipetted at a low density onto fresh selection plates.
3. Callus was bombarded while sitting directly on medium or on filters. Cells were transferred to selective medium 1-14 days after particle bombardment. Tissue was transferred on filters 1-3 times at 2 weeks intervals to fresh selective medium. Callus was then briefly put into liquid to disperse the tissue onto selective plates at a low density.
4. Callus tissue was transferred onto selective plates one to seven days after DNA introduction. Tissue was subcultured as small units of callus on selective plates until transformants were identified.

The totals demonstrate that 27 of 37 maize cultures were transformable. Of those cell lines tested 11 out of 20 have produced fertile plants and 7 are in progress. As this table indicates, transformable cultures have been produced from ten different genotypes of maize, including both hybrid and inbred varieties. These techniques for development of transformable cultures are also important in direct transformation of intact tissues, such as immature embryos as these techniques rely on the ability to select transformants in cultured cell systems.

Table 2

Genotype	Culture	Method	Transformable	Fertile Plants
A188 x B73	G(1x6)92	1	+	-
	G(1x6)716	1,2	+	+
	G(1x6)82	1	+	+
	G(1x6)98	1	-	NA
	G(1x6)99	1	-	NA
	D(1x6)122#3	2	-	NA
	D(1x6)114	2	-	NA
	D(1x6)17#33	2	In progress	In progress
	HB13-3	3	+	In progress
	HA133-227	2	-	NA
	G(6x1)17#25 C	3	+	In progress
	ABT4	4	+	+
	ABT3	4	+	+
	AB60	4	+	+
	AB61	4	+	+
	AB63	4	+	+
	AB80	4	+	+
	AB82	4	+	In progress
	ABT6	4	+	ND
	AB12	4	+	+
	PH2	4	+	+
	AB69	4	+	-
	AB44	4	+	-

Genotype	Culture	Method	Transformable	Fertile Plants
	AB62	4	+	ND
A188xB84	G(1xM)82	1	+	-
A188xH99	HJ11-7	3	+	In progress
B73xA188	G(6x1)12#7	2	-	NA
	D(6x1)11#43	2	-	NA
	E1	2	+	-
Hi-II	G(CW)31#24		+	In progress
B73	(6)91#3	2	-	NA
	(6)91#2	2	-	NA
B73-derived	AT824	1,2,3	+	+
N1017A	AZ11137a	2	+	In progress
Cat 100	CB	2	+	ND
	CC	2	+	ND
A188	E4	2	+	-

The symbol "-" indicates that the line was not transformable after 3 attempts or plants were sterile

NA indicates Not Applicable

ND indicates Not Done

#### **Example 1: Initiation of the Suspension Culture G(A188XB73)716 (designated SC716)**

##### **for Use in Transformation**

This Example describes the development of a maize suspension culture, designated SC716, which was employed in various of the transformation studies described hereinbelow. The Type II tissue used to initiate the cell suspension was initiated from immature embryos of A188 x B73 plated onto N6-based medium with 1 mg/ml 2,4-D (201; see Table 1). A Type II callus was initiated by visual

selection of fast growing, friable embryogenic cells. The suspension was initiated within 6 months after callus initiation. Tissue chosen from the callus to initiate the suspension consisted of undifferentiated Type II callus. The characteristics of this undifferentiated tissue include the earliest stages of embryo development and soft, friable, undifferentiated tissue underlying it.

Approximately one gram of tissue was added to 20 mls of liquid medium. In this example, the liquid medium was medium 402 to which different slow-release growth regulator capsule treatments were added (Adams, W.R., Adams, T.R., Wilston, H.M., Krueger, R.W., and Kausch, A.P, Silicone Capsules for Controlled Auxin Release, in preparation). These capsule treatments included 2,4-D, NAA, 2,4-D plus NAA, and two NAA capsules. One flask was initiated for each of the different 402 media plus growth regulator combinations. Every 7 days each culture was subcultured into fresh medium by transferring a small portion of the cellular suspension to a new flask. This involved swirling the original flask to suspend the cells (which tend to settle to the bottom of the culture vessel), tilting the flask on its side and allowing the denser cells and cell aggregates to settle slightly. One ml of packed cells was then drawn off from this pool of settled cells together with 4 mls of conditioned medium and added to a flask containing 20 ml fresh medium. A sterile ten ml, wide tip, pipet was used for this transfer (Falcon 7304). Any very large aggregates of cells which would not pass easily through the pipet tip were excluded. If a growth regulator capsule was present, it was also transferred to the new flask.

After approximately 7 weeks, the loose embryogenic cell aggregates began to predominate and fragment in each of the cultures, reaching a state referred to as "dispersed." The treatment which yielded the highest proportion of embryogenic clusters was the 402 medium plus one NAA capsule. After the cultures became dispersed and were doubling approximately every two to three days as determined by increase in packed cell volume, a one ml packed cell volume inoculum from each

culture was transferred into 20 ml 401 medium using a ten ml narrow tip pipet (Falcon 7551). These transfers were performed about every 3½ days. An inoculum from the 402 plus 2,4-D plus NAA capsules culture was also used to initiate a culture in 409 medium (402 without 2,4-D and including 10mg/l dicamba) either with or without 1 ml coconut water (Gibco 670-8130AG) per 25 ml culture medium.

The most dispersed cultures were cryopreserved after 2 weeks, 2 months or 5 months.

The culture grown on 409 with coconut water was thawed eight months after cryopreservation, cultured for two weeks on solid 201 culture medium using BMS as a feeder layer (Rhodes *et al.*, 1988) and transferred to media 409 without coconut water. The culture was maintained by subculturing twice weekly in 409 medium by the method described above.

**Example 2: Initiation of the Suspension Culture**  
**(A188 X B73)82 (designated SC82) for**  
**Use in Transformation**

This Example describes the development of another cell line employed in various of the transformation studies set forth below, termed SC82. In the development of SC82, inoculum for suspension culture initiation was visually selected from a Type II callus that was initiated from A188 x B73 immature embryos plated on a N6-based medium containing 13.2 mg/l dicamba (227, Table 1). The suspension culture was initiated within 3 months of initiation of the Type II callus. Small amounts (50-100 mg) of callus distinguishable by visual inspection because of its highly proembryonic morphology, were isolated from more mature or organized structures and inoculated into a 50 ml flask containing 5 mls of filter-sterilized conditioned medium from the various G(A188 x B73) 716 suspension

cultures (402 medium with four types of capsule treatments and 409 medium).

After one week, this 5 ml culture was sieved through a 710 micron mesh and used to inoculate 20 mls of corresponding fresh and filter-sterilized conditioned medium from the established G(A188 x B73) 716 cultures in 150 ml flasks. After one week or more of growth, two mls of packed cells were subcultured to fresh media by the method described above. The suspension culture maintained on 409 by this method was then cryopreserved within 3 months. The original cell line, which was maintained on 409 (not a reinoculated cryopreserved culture) was used in experiments 1 and 2 months later which resulted in stable transformation and selection (see Table 6 below). The cryopreserved culture was used for experiment 6 (see Table 6 below).

### **Example 3 : Initiation and Maintenance of Cell Line AT824.**

This example describes the initiation and maintenance of cell line AT824 which has been used routinely for transformation experiments. Immature embryos (0.5 - 1.0mm) were excised from the B73-derived inbred line AT and cultured on N6 medium with 100 uM silver nitrate, 3.3 mg/L dicamba, 3% sucrose and 12 mM proline (2004). Six months after initiation type I callus was transferred to medium 2008. Two months later type I callus was transferred to a medium with a lower concentration of sucrose (279). A sector of type II callus was identified 17 months later and was transferred to 279 medium. This cell line is uniform in nature, unorganized, rapid growing, and embryogenic. This culture is desirable in the context of this invention as it is easily adaptable to culture in liquid or on solid medium.

The first suspension cultures of AT824 were initiated 31 months after culture initiation. Suspension cultures may be initiated in a variety of culture media including media containing 2,4-D as well as dicamba as the auxin source, e.g.,



media designated 210, 401, 409, 279. Cultures are maintained by transfer of approximately 2 ml packed cell volume to 20 ml fresh culture medium at 3 ½ day intervals. AT824 can be routinely transferred between liquid and solid culture media with no effect on growth or morphology.

5

Suspension cultures of AT824 were initially cryopreserved 33-37 months after culture initiation. The survival rate of this culture was improved when it was cryopreserved following three months in suspension culture. AT824 suspension cultures have been cryopreserved and reinitiated from cryopreservation at regular intervals since the initial date of freezing. Repeated cycles of freezing have not affected the growth or transformability of this culture.

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**Example 4: Initiation and maintenance of cell lines ABT3, ABT4, ABT6, AB80, AB82, AB12, AB44, AB60, AB61, AB62, AB63, AB69**

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Friable, embryogenic maize callus cultures were initiated from hybrid immature embryos produced by pollination of inbred A188 plants (University of Minnesota, Crop Improvement Association) with pollen of inbred line B73 plants (Iowa State University). Ears were harvested when the embryos had reached a length of 1.5 to 2.0 mm. The whole ear was surface sterilized in 50% v/v commercial bleach (2.63% w/v sodium hypochlorite) for 20 min. at room temperature. The ears were then washed with sterile distilled, deionized water. Immature embryos were aseptically isolated and placed on nutrient agar initiation/maintenance media with the root/shoot axis exposed to the medium.

20

Initiation/maintenance medium (hereinafter referred to as medium 734) consisted of N6 basal medium (Chu 1975) with 2% (w/v) sucrose, 1.5 mg per liter 2,4-dichlorophenoxyacetic acid (2,4-D), 6 mM proline, and 0.25% Gelrite (Kelco, Inc. San Diego). The pH was adjusted to 5.8 prior to autoclaving. Unless otherwise stated, all tissue culture manipulations were carried out under sterile conditions.

25

30

The immature embryos were incubated at 26°C in the dark. Cell proliferation from the scutellum of the immature embryos were evaluated for friable consistency and the presence of well defined somatic embryos. Tissue with this morphology was transferred to fresh media 10 to 14 days after the initial plating of the immature embryos. The tissue was then subcultured on a routine basis every 14 to 21 days. Sixty to eighty milligram quantities of tissue were removed from pieces of tissue that had reached a size of approximately one gram and transferred to fresh medium. Subculturing always involved careful visual monitoring to be sure that only tissue of the correct morphology was maintained. The presence of somatic embryos ensured that the cultures would give rise to plants under the proper conditions. The cell cultures named ABT3, ABT4, ABT6, AB80, AB82, AB12, AB44, AB60, AB61, AB62, AB63, AB69 were initiated in this manner. The cell lines ABT3, ABT4, and ABT6 were initiated from immature embryos of a 5-methyltryptophan resistant derivative of A188 x B73.

#### **Example 5: Initiation and Maintenance of type II callus of the genotype Hi-II**

The Hi-II genotype of corn was developed from an A188 x B73 cross. This genotype was developed specifically for a high frequency of initiation of type II cultures (100% response rate, Armstrong et al., 1991). Immature embryos (8-12 days post-pollination, 1 to 1.2 mm) were excised and cultured embryonic axis down on N6 medium containing 1 mg/L 2,4-D, 25 mM L-proline (201) or N6 medium containing 1.5 mg/L 2,4-D, 6mM L-proline (734). Type II callus can be initiated either with or without the presence of 100  $\mu$ M AgNO<sub>3</sub>. Cultures initiated in the presence of AgNO<sub>3</sub> was transferred to medium lacking this compound 14-28 days after culture initiation. Callus cultures were incubated in the dark at 23-28°C and transferred to fresh culture medium at 14 day intervals.

Hi-II type II callus is maintained by manual selection of callus at each transfer. Alternatively, callus can be resuspended in liquid culture medium, passed

through a 1.9 mm sieve and replated on solid culture medium at the time of transfer. It is believed that this sequence of manipulations is one way to enrich for recipient cell types. Regenerable type II callus that is suitable for transformation can be routinely developed from the Hi-II genotype and hence new cultures are developed every 6-9 months. Routine generation of new cultures reduces the period of time over which each culture is maintained and hence insures reproducible, highly regenerable, cultures that routinely produce fertile plants.

#### **Example 6: Initiation of Cell Line E1**

An ear of the genotype B73 was pollinated by A188. Immature embryos (1.75- 2.00 mm) were excised and cultured on 212 medium (see Table 1). About 4 months after embryo excision, approximately 5 ml PCV type II callus was inoculated into 50 ml liquid 210 medium (see Table 1). The suspension was maintained by transfer of 5 ml suspension to 50 ml fresh 210 medium every 3 1/2 days. This suspension culture was cryopreserved about 4 months after initiation.

#### **D. Cryopreservation Methods**

Cryopreservation is important because it allows one to maintain and preserve a known transformable cell culture for future use, while eliminating the cumulative detrimental effects associated with extended culture periods.

Cell suspensions and callus were cryopreserved using modifications of methods previously reported (Finkle, 1985; Withers & King, 1979). The cryopreservation protocol comprised adding a pre-cooled (0°C) concentrated cryoprotectant mixture stepwise over a period of one to two hours to pre-cooled (0°C) cells. The mixture was maintained at 0°C throughout this period. The volume of added cryoprotectant was equal to the initial volume of the cell suspension (1:1 addition), and the final concentration of cryoprotectant additives

was 10% dimethyl sulfoxide, 10% polyethylene glycol (6000 MW), 0.23 M proline and 0.23 M glucose. The mixture was allowed to equilibrate at 0°C for 30 minutes, during which time the cell suspension/ cryoprotectant mixture was divided into 1.5 ml aliquot (0.5 ml packed cell volume) in 2 ml polyethylene cryo-vials. The tubes were cooled at 0.5°C/minute to -8°C and held at this temperature for ice nucleation.

Once extracellular ice formation had been visually confirmed, the tubes were cooled at 0.5°C/minute from -8°C to -35°C. They were held at this temperature for 45 minutes (to insure uniform freeze-induced dehydration throughout the cell clusters). At this point, the cells had lost the majority of their osmotic volume (i.e. there is little free water left in the cells), and they could be safely plunged into liquid nitrogen for storage. The paucity of free water remaining in the cells in conjunction with the rapid cooling rates from -35 to -196°C prevented large organized ice crystals from forming in the cells. The cells are stored in liquid nitrogen, which effectively immobilizes the cells and slows metabolic processes to the point where long-term storage should not be detrimental.

Thawing of the extracellular solution was accomplished by removing the cryo-tube from liquid nitrogen and swirling it in sterile 42°C water for approximately 2 minutes. The tube was removed from the heat immediately after the last ice crystals had melted to prevent heating the tissue. The cell suspension (still in the cryoprotectant mixture) was pipetted onto a filter, resting on a layer of BMS cells (the feeder layer which provided a nurse effect during recovery). Dilution of the cryoprotectant occurred slowly as the solutes diffused away through the filter and nutrients diffused upward to the recovering cells. Once subsequent growth of the thawed cells was noted, the growing tissue was transferred to fresh culture medium. The cell clusters were transferred back into liquid suspension medium as soon as sufficient cell mass had been regained (usually within 1 to 2 weeks). After the culture was reestablished in liquid (within 1 to 2 additional

weeks), it was used for transformation experiments. When desired, previously cryopreserved cultures may be frozen again for storage.

#### **E. DNA Segments Comprising Exogenous Genes**

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As mentioned previously, there are several methods to construct the DNA segments carrying DNA into a host cell that are well known to those skilled in the art. The general construct of the vectors used herein are plasmids comprising a promoter, other regulatory regions, structural genes, and a 3' end.

10

Several plasmids encoding a variety of different genes have been constructed by the present inventors, the important features of which are represented below in Table 3. Certain of these plasmids are also shown in Figure 1: pDPG165, Fig. 1 (A, C); pDPG208, Fig. 1 (B, D); pDPG141, Fig. 1 (E);  
15 pDPG237, Fig. 1 (F); pDPG313 through pDPG319, Fig. 1 (H) through Fig. 1 (N); pDPG290, Fig. 1 (O); pDPG300 through pDPG304, Fig. 1 (P) through Fig. 1 (S); pDPG386 through pDPG389, Fig. 1 (T) through Fig. 1 (W); pDPG140, Fig. 1(X); pDPG172, Fig. 1(Y); pDPG425, Fig. 1(Z); pDPG427, Fig. 1(AA); pDPG451, Fig. 1 (BB); pDPG 354, Fig 1 (CC); pDPG344, Fig 1 (DD); pDPG337, Fig 1 (EE).

20

**TABLE 3**

5	RECOMBINANT VECTOR DESIGNATION & SOURCE	PARENT REPLICON	INSERT DNA	DELIBERATE EXPRESSION ATTEMPT
	pDPG140	pUC19	1,118,102, 103	1
10	pDPG141	pUC19	1, 100, 101	1
	pDPG165	pUC19	2, 100, 101	2
	pDPG172	pUC19	3,118,102, 103	3
	pDPG182	pUC19	2,118,102, 103	2
	pDPG205	pVK101	1, 101, 102	1
15	pDPG208	pUC19	1, 100	1
	pDPG215	pUC18	3, 100, 102, 103	3
	pDPG215	pUC19	3, 100, 101, 102	3
	pDPG226	pUC19	4, 100, 101	4
20	pDPG230-231, 251, 262- 264, 279, 282, 283	pUC19	1, 2, 100, 101	1, 2
	pDPG238-239	pUC19	2, 4, 100, 101	2, 4
	pDPG240-241	pUC19	2, 5, 100, 101	2, 5
	pDPG243-244	pUC19	2, 6, 100, 101	2, 6
	pDPG246	pUC19	7, 100, 101	7

	RECOMBINANT VECTOR DESIGNATION & SOURCE	PARENT REPLICON	INSERT DNA	DELIBERATE EXPRESSION ATTEMPT
	pDPG265	pBR325	9, 100	9
	pDPG266-267	pUC19	8, 100, 101	8
	pDPG268-269	pUC19	1, 100, 102	1
	pDPG270-273	pUC19	1, 100, 101	1
5	pDPG274	pUC19	9, 100, 101	9
	pDPG275	pUC18	10, 3, 100	10, 3
	pDPG287	pUC19	2, 103, 105	2
	pDPG288	pUC13	11, 100, 103	11
	pDPG290	pUC19	12, 100, 103	12
10	pDPG291	pUC19	1, 103, 104	1
	pDPG300	PUC19	2, 12, 100, 101, 102, 103	2, 12
	pDPG301	pUC19	2, 12, 100, 101, 102, 103	2, 12
	pDPG302	pUC18	2, 12, 100, 101, 102, 103	2, 12
	pDPG303	pUC18	2, 12, 100, 101, 102, 103	2, 12
15	pDPG304	ColE1	13, 100, 103	13
	pDPG313	pUC18	2, 4, 100, 103, 106, 107	2, 4

	RECOMBINANT VECTOR DESIGNATION & SOURCE	PARENT REPLICON	INSERT DNA	DELIBERATE EXPRESSION ATTEMPT
	pDPG314	pUC19	2, 4, 100, 103, 106, 107	2, 4
	pDPG315	pUC23	2, 4, 100, 103, 106, 108	2, 4
	pDPG316	pUC23	2, 4, 100, 103, 106, 108	2, 4
	pDPG317	pUC23	2, 4, 100, 103, 106, 107	2, 4
5	pDPG318	pUC23	2, 4, 100, 103, 106, 109	2, 4
	pDPG319	pUC23	2, 4, 100, 103, 106, 109	2, 4
	pDPG320	pUC18	11, 100, 102, 103	11
	pDPG324	pUC19	1, 2, 100, 101	1, 2
	pDPG325	pUC19	1, 2, 100, 101	1, 2
10	pDPG326	pUC19	1, 2, 100, 101, 102	1, 2
	pDPG327	pUC19	1, 2, 100, 101, 102	1, 2
	pDPG328	pUC19	1, 2, 100, 101, 113	1, 2
	pDPG329	pUC19	1, 2, 100, 101, 113	1, 2



	RECOMBINANT VECTOR DESIGNATION & SOURCE	PARENT REPLICON	INSERT DNA	DELIBERATE EXPRESSION ATTEMPT
	pDPG332	pUC19	14, 100, 110	14
	pDPG334	pGEM3	15, 111, 121, 103	15
	pDPG335	pUC119	15, 112, 122, 114	15
	pDPG336	pIC20H	19, 100, 117, 103	19
5	pDPG337	pIC20H	19, 100, 117, 103	19
	pDPG338	pUC120	16, 112, 114, 115	16
	pDPG339	pUC119	17, 111, 103	17
	pDPG340	pUC19	18, 111, 103	18
	pDPG344	pSK	11, 100, 102, 110	11
10	pDPG345	pSK	2, 14, 100, 102, 110, 103	2, 14
	pDPG346	pSK	2, 14, 100, 102, 110, 103	2, 14
	pDPG347	pSK	2, 14, 100, 102, 110, 103	2, 14
	pDPG348	pSK	2, 14, 100, 102, 110, 103	2, 14
	pDPG351	pUC19	3, 100, 101	3

	RECOMBINANT VECTOR DESIGNATION & SOURCE	PARENT REPLICON	INSERT DNA	DELIBERATE EXPRESSION ATTEMPT
	pDPG354	pSK-	19, 100, 101, 110	19
	pDPG355	pUC19	1, 100, 102, 103	1
	pDPG356	pUC19	1, 100, 116, 103	1
	pDPG357	pIC20H	1, 100, 117, 103	1
5	pDPG358	pUC8	1, 118, 102, 103	1
	pDPG359	pUC119	1, 119, 103	1
	pDPG360	pUC119	1, 120, 103	1
	pDPG361	pUC19	1, 111, 103	1
	pDPG362	pUC120	1, 112, 103	1
10	pDPG363	pSP73	2, 100, 102, 103	2
	pDPG364	pUC119N	20, 100	20
	pDPG365	pUC19	21, 100, 103	21
	pDPG366	pSP73	22, 100, 102, 103	22
	pDPG367	pBS +	22, 100, 102, 103	22
15	pDPG368	pSP73	24, 100, 102, 103	24
	pDPG369	pIC20H	24, 100, 117, 103	24
	pDPG370	pBS +	5, 100, 116, 103	5

	RECOMBINANT VECTOR DESIGNATION & SOURCE	PARENT REPLICON	INSERT DNA	DELIBERATE EXPRESSION ATTEMPT
	pDPG371	pSP73	15, 100, 121, 103	15
	pDPG372	pUC119	15, 100, 122, 103	15
	pDPG373	pUC119	16, 120, 115	16
	pDPG374	pUC120	16, 112, 114	16
5	pDPG375	pUC119	16, 111, 115	16
	pDPG376	pUC119	18, 111, 103	18
	pDPG377	pUC119	18, 111, 103	18
	pDPG378	pUC119	17, 111, 103	17
	pDPG379	pUC119	17, 111, 103	17
10	pDPG380	pUC119	17, 111, 103	17
	pDPG381	pIC20H	19, 100, 117, 103	19
	pDPG382	pIC20H	19, 100, 117, 103	19
	pDPG384	pUC8	19, 100, 102, 103	19
	pDPG385	pSP73	23, 100, 121, 103	23
15	pDPG386	pACYC177	6,114	6
	pDPG387	pACYC177	1,6,100,114	1,6

	RECOMBINANT VECTOR DESIGNATION & SOURCE	PARENT REPLICON	INSERT DNA	DELIBERATE EXPRESSION ATTEMPT
	pDPG388	pACYC177	2,6,100,103 , 114	2,6
	pDPG389	pACYC177	2,6,100,103 , 114,	2,6
	pDPG391	pUC8	19, 100, 102, 103	19
	pDPG392	pUC19	2, 100, 101	2
5	pDPG393	pSK-	4, 109, 102, 106, 103	4
	pDPG394	pSK-	4, 124, 106, 103	4
	pDPG396	pBS +	19, 100, 102, 7, 103, 121	19
	pDPG404	pSK-	1, 108, 103	1
	pDPG405	pSK-	1, 107, 103	1
10	pDPG406	pSK-	1, 109, 103	1
	pDPG407	pSK-	1, 109, 102, 103	1
	pDPG408	pSK-	1, 124, 103	1
	pDPG411	pBS +	19, 100, 103, 121	19
	pDPG415	pUC8	19, 118, 103	19
15	pDPG418	pGEM	15, 122, 123	15
	pDPG419	pUC19	3,100,101,1 16,	3
	pDPG420	pUC19	3,100,101,1 16	3

	RECOMBINANT VECTOR DESIGNATION & SOURCE	PARENT REPLICON	INSERT DNA	DELIBERATE EXPRESSION ATTEMPT
	pDPG421	pSP72	2,103,126	2
	pDPG422	pBS	1,103,126	1
	pDPG424	pSK-	4, 124, 102, 103	4
	pDPG427	pSK-	25, 124, 102, 103	25
5	pDPG434	pSK-	25,103,126	25
	pDPG436	pSK-	25,100,102, 103	25
	pDPG437	pUC19	2.100,101,1 02	2
	pDPG438	pUC19	2,100,101,1 02	2
	pDPG439	pUC19	2,100,101,1 02	2
10	pDPG441	pSK-	25,103,108	25
	pDPG443	pSK-	25,100,103, 108	25
	pDPG447	pSK-	25,102,103, 109	25
	pDPG451	pUC18	26,100,102, 103	26
	pDPG452	pGEM	2,123	2
15	pDPG453	pUC19	2, 103,127	2
	pDPG456	pSK-	19, 100, 101, 110	19
	pDPG457	pSK-	19, 100, 101, 110	19
	pDPG458	pSK-	1,103,127	1

RECOMBINANT VECTOR DESIGNATION & SOURCE	PARENT REPLICON	INSERT DNA	DELIBERATE EXPRESSION ATTEMPT
pDPG465	pSK-	25,100,101, 102	25
pDPG467	pSK-	25,102,118	25
pDPG469	pUC19	26,118,102, 103	26
pDPG474	pUC19	27,100,102, 103	27

5

## KEY:

### Insert DNA and Deliberate Expression Attempt

- 5 1. The *uidA* gene from *E. Coli* encodes  $\beta$ -glucuronidase (GUS). Cells expressing *uidA* produce a blue color when given the appropriate substrate. Jefferson, R.A. 1987. Plant Mol. Biol. Rep 5: 387-405.
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- 15 3. The *lux* gene from firefly encodes luciferase. Cells expressing *lux* emit light under appropriate assay conditions. deWet, J.R., Wood, K.V., DeLuca, M., Helinski, D.R., Subramani, S. 1987. Mol. Cell. Biol. 7: 725-737.
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- 25 5. The *dhfr* gene from mouse encodes dihydrofolate reductase (DHFR). Cells expressing *dhfr* are resistant to methotrexate. Eichholtz, D.A., Rogers, S.G., Horsch, R.B., Klee, H.J., Hayford, M., Hoffman, N.L., Bradford, S.B., Fink, C., Flick, J., O'Connell, K.M., Frayley, R.T. 1987. Somatic Cell Mol. Genet. 13: 67-76.
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- 40 8. The *xyIE* gene from *Ps. putida* encodes catechol dihydroxygenase. Cells expressing *xyIE* produce a chromogenic compound when given the appropriate substrate. Zukowsky et al. 1983. Proc. Nat. Acad. Sci. USA 80: 1101-1105.
9. The R,C1 and B genes from maize encode proteins that regulate anthocyanin biosynthesis in maize. Goff, S., Klein, T., Ruth, B., Fromm, M., Cone, K., Radicella, J., Chandler, V. 1990. EMBO J.: 2517-2522.

10. The *als* gene from *Zea mays* encodes acetolactate synthase. The enzyme was mutated to confer resistance to sulfonylurea herbicides. Cells expressing *als* are resistant to the herbicide Gleen. Yang, L.Y., Gross, P.R., Chen, C.H., Lissis, M. 1992. Plant Molecular Biology 18: 1185-1187.
11. The proteinase inhibitor II gene was cloned from potato and tomato. Plants expressing the proteinase inhibitor II gene show increased resistance to insects. Potato: Graham, J.S., Hall, G., Pearce, G., Ryan, C.A. 1986 Mol. Cell. Biol. 2: 1044-1051. Tomato: Pearce, G., Strydom, D., Johnson, S., Ryan, C.A. 1991. Science 253: 895-898.
12. The *Bt* gene from *Bacillus thuringiensis* berliner 1715 encodes a protein that is toxic to insects. Plants expressing this gene are resistant to insects. This gene is the coding sequence of *Bt* 884 modified in two regions for improved expression in plants. Vaeck, M., Reynaerts, A., Hofte, H., Jansens, S., DeBeuckeleer, M., Dean, C., Aeabeau, M., Van Montagu, M., and Leemans, J. 1987. Nature 328: 33-37.
13. The *bxn* gene from *Klebsiella ozaenae* encodes a nitrilase enzyme specific for the herbicide bromoxynil. Cells expressing this gene are resistant to the herbicide bromoxynil. Stalker, D.m., McBride, K.E., and Malyj, L. Science 242: 419-422, 1988.
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17. The A20 sequence encodes the 19kd zein storage protein of *Zea mays*. Expression of the construct in maize alters quantities of the native 19kd zein gene.
18. The Z4 sequence is for the 22kd zein storage protein of *Zea mays*. Expression of this construct in maize alters quantities of the native 22kd zein gene.



19. The Bt gene cloned from *Bacillus thuringiensis* Kurstaki encodes a protein that is toxic to insects. The gene is the coding sequence of the cry IA(c) gene modified for improved expression in plants. Plants expressing this gene are resistant to insects. Höfte, H. and Whiteley, H.R., 1989. Microbiological Reviews. 53: 242-255.
20. The als gene from *Arabidopsis thaliana* encodes a sulfonylurea herbicide resistant acetolactate synthase enzyme. Cells expressing this gene are resistant to the herbicide Gleen. Haughn, G.W., Smith, J., Mazur, B., and Somerville, C. 1988. Mol. Gen. Genet. 211: 266-271.
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23. The *lysC* gene from *E. coli* encodes the enzyme aspartyl kinase III. Expression of this gene leads to increased levels of lysine in cells.
24. The hygromycin phosphotransferase II gene was isolated from *Streptomyces hygroscopicus*. Expression of this gene in cells produces resistance to the antibiotic hygromycin.
25. The EPSPS gene (5-enolpyruvylshikimate - 3-phosphate synthase) gene from Zea Mays was mutated to confer resistance to the herbicide Roundup® (glyphosate). An isoleucine has been substituted for threonine at amino acid position 102 and a serine has been substituted for proline at amino acid position 106.
26. The *mtlD* gene was cloned from *E. coli*. This gene encodes the enzyme mannitol-1-phosphate dehydrogenase. Lee and Saier, 1983. J. of Bacteriol. 153:685.
27. The HVA-1 gene encodes a Late Embryogenesis Abundant (LEA) protein. This gene was isolated from barley. Dure, L., Crouch, M., Harada, J., Ho, T.-H. D. Mundy, J., Quatrano, R, Thomas, T, and Sung, R., Plant Molecular Biology 12: 475-486.

## Regulatory Sequences

- 5 100. Promoter sequences from the Cauliflower Mosaic Virus genome. Odell, J.T., Nagy, F., and Chua, N.-H. 1985. *Nature* 313: 810-812.
- 10 101. Promoter and terminator sequences from the Ti plasmid of *Agrobacterium tumefaciens*. (a) Bevan, M., 1984. *Nucleic Acid Research* 12: 8711-8721; (b) Ingelbrecht, I.L.W., Herman, L.M.F., DeKeyser, R.A., Van Montagu, M.C., Depicker, A.G. 1989. *The Plant Cell* 1: 671-680; (c) Bevan, M., Barnes, W.M., Chilton, M.D., 1983. *Nucleic Acid Research*. 11: 369-385; (d) Ellis, J.G., Llewellyn, D.J., Walker, J.C., Dennis, E.S., Peacocu, W.J. 1987. *EMBO J.* 6: 3203-3208.
- 15 102. Enhancer sequences from the maize alcohol dehydrogenase gene. Callis, J., Fromm, M.E., Walbot, V., 1987. *Genes Dev.* 1: 1183-1200.
- 20 103. Terminator sequences from Ti plasmid of *Agrobacterium tumefaciens*. (a) Bevan, M., 1984. *Nucleic Acid Research* 12: 8711-8721; (b) Ingelbrecht, I.L.W., Herman, L.M.F., DeKeyser, R.A., Van Montagu, M.C., Depicker, A.G. 1989. *The Plant Cell* 1: 671-680; (c) Bevan, M., Barnes, W.M., Chilton, M.D., 1983. *Nucleic Acid Research*. 11: 369-385.
- 25 104. Pollen specific promoter sequence ZM13 from maize.
105. Transit peptide sequence from *rbcS* gene from pea. Fluhr, R., Moses, P., Morelli, G., Coruzzi, C., Chua, N.-H. 1986. *EMBO J.* 5: 2063-2071.
- 30 106. Optimized transit peptide sequence consisting of sequences from sunflower and maize. Constructed by Rhône Poulenc Agrochimie.
- 35 107. Fused promoter sequences from Cauliflower Mosaic Virus genome and *Arabidopsis thaliana* H4 histone gene. Constructed by Rhône Poulenc Agrochimie.
108. Promoter sequence from *Arabidopsis thaliana* histone H4 gene. Chaboute, H.E., Chambet, N., Philipps, G., Ehling, M. and Grigot, C. 1987. *Plant Mol. Biol.* 8: 179-191.
- 40 109. Promoter sequence from maize  $\alpha$ -tubulin gene.
110. Terminator sequences from the potato proteinase inhibitor II gene. An, G., Mitra, A., Choi, H.K., Costa, M.A., An, K., Thornburg, R.W., Ryan, C.A. 1989. *Plant Cell* 1: 115-122.

111. Promoter from the maize 10kd zein gene.
112. Promoter from the maize 27kd zein gene. Ueda, T. and Messing, J. 1991. Theor. Appl. Genet. 82: 93-100.
- 5 113. The matrix attachment region (MAR) was isolated from chicken. Use of this DNA sequence reduces variations in gene expression due to integration position effects. Stief, A., Winter, D., Stratling, W.H., Sippel, A.E. 1989. Nature 341: 343.
- 10 114. Terminator sequences from the Cauliflower Mosaic Virus genome. Timmermans, M.C.P., Maliga, P., Maliga, P., Vieira, J. and Messing, J. 1990. J. Biotechnol. 14: 333-344.
- 15 115. Terminator from maize 10kd zein gene. Kirihaara, J.A., Hunsperger, J.P., Mahoney, W.C., Messing, J. 1988. Mol. Gen. Genet. 211: 477-484.
- 20 116. Enhancer sequence from the shrunken-1 gene of *Zea mays*. Vasil, V., Clancy, M., Ferl, R.J., Vasil, I.K., Hannah, L.C. 1989. Plant Physiol. 91: 1575-1579.
- 25 117. RNA leader sequence from the ribulose-bis-phosphate carboxylase gene from *Glycine max*. Joshi, C.P. 1987. Nucleic Acids Res. 15:6643-9640.
118. Promoter sequence from the alcohol dehydrogenase gene of *Zea mays*. Walker, J.C., Howard, E.A., Dennis, E.S., Peacock, W.J. 1987. P.N.A.S. 84: 6624-6628.
- 30 119. Promoter sequence from the glutamine synthetase gene of *Zea mays*.
120. Promoter sequence from the 22kD (Z4) zein gene of *Zea mays*. Schmidt, R.J., Ketudat, M., Ankerman, M.J. and Hoschek, G. 1992. Plant Cell 4: 689-700.
- 35 121. Transit peptide sequence of the ribulose bis-phosphate carboxylase small subunit gene from *Zea mays*. GenBank Accession Y00322.
- 40 122. Transit peptide sequence of the dihydropicolinic acid synthase gene of *Zea mays*.
123. Globulin-1, *glb1*, promoter and terminator sequences from *Zea mays*. Belanger and Kriz. 1991

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126. Actin-1 5' region including promoter from *Zea mays*. Wang, Y., Zhang, W., Cao, J., McEhoy, D. and Ray Wu. 1992. Molecular and Cellular Biology 12: 3399-3406.
127. The DS element isolated from *Zea mays*.

DNA segments encoding the *bar* gene were constructed into a plasmid, termed pDPG165, which was used to introduce the bialaphos resistance gene into recipient cells (see Figures 1A and C). The *bar* gene was cloned from *Streptomyces hygroscopicus* (White et al., 1990) and exists as a 559-bp Sma I fragment in plasmid pIJ4101. The sequence of the coding region of this gene is identical to that published (Thompson *et al.*, 1987). To create plasmid pDPG165, the Sma I fragment from pIJ4104 was ligated into a pUC19-based vector containing the Cauliflower Mosaic Virus (CaMV) 35S promoter (derived from pBI221.1. provided by R. Jefferson, Plant Breeding Institute, Cambridge, England), a polylinker, and the transcript 7 (Tr7) 3' end from *Agrobacterium tumefaciens* (3' end provided by D. Stalker, Calgene, Inc., Davis, CA).

An additional vector encoding GUS, pDPG208, (Figures 1B and 1D) was used in these experiments. It was constructed using a 2.1 kb BamHI/EcoRI fragment from pAGUS1 (provided by J. Skuzeski, University of Utah, Salt Lake City, UT) containing the coding sequence for GUS and the *nos* 3'-end from *Agrobacterium tumefaciens*. In pAGUS1 the 5'-noncoding and 5'-coding sequences for GUS were modified to incorporate the Kozak consensus sequence (Kozak, 1984) and to introduce a new HindIII restriction site 6 bp into the coding region of the gene (see Figure 1E). The 2.1 kb BamHI/EcoRI fragment from

pAGUS1 was ligated into a 3.6 kb BamHI/EcoRI fragment of a pUC19-based vector pCEV1 (provided by Calgene, Inc., Davis, CA). The 3.6 kb fragment from pCEV1 contains pUC19 and a 430 bp 35S promoter from cauliflower mosaic virus adjacent to the first intron from maize *Adh1*.

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In terms of a member of the R gene complex for use in connection with the present invention, the most preferred vectors contain the 35S promoter from Cauliflower Mosaic Virus, the first intron from maize *Adh1* gene, the Kozak consensus sequence, Sn:bol3 cDNA, and the transcript 7' 3' end from *Agrobacterium tumefaciens*. One such vector prepared by the inventors is termed pDPG237. To prepare pDPG237 (see Figure 1F), the cDNA clone of Sn:bol3 was obtained from S. Dellaporta (Yale University, USA). A genomic clone of Sn was isolated from genomic DNA of Sn:bol3 which had been digested to completion with HindIII, ligated to lambda arms and packaged in vitro. Plaques hybridizing to two regions of cloned R alleles, R-nj and R-sc (Dellaporta *et al.*, 1988) were analyzed by restriction digest. A 2 kb Sst-HincII fragment from the pSn7.0 was used to screen a cDNA library established in lambda from RNA of light-irradiated scutellar nodes of Sn:bol3. The sequence and a restriction map of the cDNA clone was established.

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The cDNA clone was inserted into the same plant expression vector described for pDPG165, the bar expression vector (see above), and contains the 35S Cauliflower mosaic virus promoter, a polylinker and the transcript 7' 3' end from *Agrobacterium tumefaciens*. This plasmid, pDPG232, was made by inserting the cDNA clone into the polylinker region; a restriction map of pDPG232 is shown in Figure 1G. The preferred vector, pDPG237, was made by removing the cDNA clone and Tr7' 3' end from pDPG232, with Aval and EcoRI and ligating it with a BamHI/EcoRI fragment from pDPG208. The ligation was done in the presence of a BamHI linker as follows (upper strand, ~~seq id no:3~~; lower strand, ~~seq id no:4~~):

SEQ ID NO:3

SEQ ID NO:4

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GATCCGTCGACCATGGCGCTTCAAGCTTC

GCAGCTGGTACCGCGAAGTTCTGAAGGGCT

The final construct of pDPG237 contained a Cauliflower mosaic virus 35S promoter, the first intron of *Adh1*, Kozak consensus sequence, the BamHI linker, cDNA of *Sn:Bo13*, and the Tr7 3' end and is shown in Figure 1F.

Additional vectors have been prepared using standard genetic engineering techniques. For example, a vector, designated pDPG128, has been constructed to include the *neo* coding sequence (neomycin phosphotransferase (APH(3')-II)).

Plasmid pDPG128 contains the 35S promoter from CaMV, the neomycin phosphotransferase gene from Tn5 (Berg *et al.*, 1980) and the Tr7 terminator from *Agrobacterium tumefaciens*. Another vector, pDPG154, incorporates the crystal toxin gene and was also prepared by standard techniques. Plasmid pDPG154 contains the 35S promoter, the entire coding region of the crystal toxin protein of *Bacillus thuringiensis* var. *kurstaki* HD 263, and the Tr7 promoter.

Various tandem vectors have also been prepared. For example, a *bar/aroA* tandem vector (pDPG238) was constructed by ligating a blunt-ended 3.2 kb DNA fragment containing a mutant EPSP synthase *aroA* expression unit (Barkai-Golan *et al.*, 1978) to NdeI-cut pDPG165 that had been blunted and dephosphorylated (NdeI introduces a unique restriction cut approximately 200 bp downstream of the Tr7 3'-end of the *bar* expression unit). Transformants having *aroA* in both orientations relative to *bar* were identified.

Additional *bar* gene vectors employed are pDPG284 and pDPG313-pDPG319 (Figure 1 H-N), the latter series being obtained from Rhône-Poulenc Agrochimie (RPA). The orientation of the *bar* gene in DPG165 was inverted with respect to the pUC vector, to obtain pDPG284. An additional 32 bp of DNA has been inserted into the NdeI site of pDPG165 and pDPG284, to obtain pDPG295 and pDPG297 and pDPG298, respectively. This extra 32 bp of sequence preserves the unique

NdeI site in each vector and adds four additional restriction sites with the following orientation, relative to the unique NarI site:

NarI - 52 bp - HpaI - NotI - NruI - EcoRI - NdeI.

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Tandem *bar-aroA* vectors with a 35S-histone promoter (constitutive and meristem enhanced expression) in convergent (pDPG314), divergent (pDPG313), and colinear orientations (pDPG317) have also been employed, as have *aroA* vectors with a histone promoter (meristem-specific promoter, (pDPG315/pDPG316) and an  $\alpha$ -tubulin promoter (root-specific, pDPG318/pDPG319) in colinear and divergent orientations to *bar* respectively.

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Plasmid pDPG290 incorporates the modified Bt *CryIA(b)* gene, *IAb6*, obtained from Plant Genetic Systems, PGS. A 1.8kb *NcoI-NheI* fragment containing *IAb6* was ligated together with the Tr7 3' region on a 0.5 Kb *NheI-EcoRI* fragment from pDPG145 and 3.6 Kb *NcoI-EcoRI* fragment from pDGP208 containing the 35S promoter, *Adh1* intron I, and the required plasmid functions to create pDPG290 (Figure 1, O).

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In the construction of *IAb6-bar* tandem vectors, modifications were made to the separate *bar* and *IAb6* expression units so that *IAb6* could be inserted as a NotI-flanked cassette into the *bar*-containing plasmid. A NotI recognition site was introduced through a series of ligations into plasmids which contained the *bar* expression unit in two orientations with respect to the new NotI recognition site. The *bar* expression unit was removed from pDPG165 as a HindIII-EcoRI fragment and ligated to HindIII-EcoRI cut pUC18 to give the plasmid pDPG284. This reversed the orientation of the *bar* expression unit with respect to the pUC plasmid. An oligonucleotide containing HpaI, PmlI, NruI, EcoRI, and NdeI restriction sites was ligated to NdeI cut pDPG165 and pDPG284 to produce pDPG285/pDPG286 and pDPG292/pDPG293, respectively. These pairs of plasmids were the two

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orientations of the oligonucleotide. These four plasmids were then cut with PmlI in order to introduce a NotI linker which destroyed the PmlI recognition site. Plasmids pDPG292 and pDPG293 containing the NotI linker were designated pDPG298 and pDPG297, respectively. A NotI site was introduced adjacent to the 3' end of the Tr7 3' region by removing a 200 bp EcoRI fragment from pDPG294. This vector was designated pDPG296.

An *IAb6* vector with flanking NotI sites was constructed via a 4-way ligation consisting of the Tr7 3' region (as a 500bp HpaI-BglII fragment from pDPG296), the *IAb6* gene (as a 1.8Kb BamHI-NcoI fragment from pDPG290), the 35S promoter-*Adh1* intron 1 cassette (as a 800bp XbaI-NcoI fragment from pDPG208), and the pBluescript II SK(-) plasmid (as a 2.95 Kb XbaI-HindII fragment). The pBluescript plasmid has a polylinker that positioned a second NotI recognition site next to the 5' end of the *IAb6* expression unit. The plasmid which contained the NotI-flanked *IAb6* expression unit was designated pDPG299. The *IAb6* expression unit was excised from pDPG299 as a 3.1kb NotI fragment and ligated to NotI cut pDPG295 and pDPG298 to yield pDPG300/pDPG301 and pDPG302/pDPG303, respectively (Figure 1 P-S). The pairs are the two orientations possible for each ligation.

A plasmid DNA, pDPG310, was constructed that contains a *bar* expression unit and a single copy of the matrix attachment region from the chicken lysozyme gene. The nuclear matrix attachment region (MAR, or "A-element") from the chicken genomic DNA region 5' to the lysozyme gene is contained on a 2.95 kb KpnI-PstI fragment in plasmid pUC19 B1-X1 (received from A.E. Sippel, Freiburg). It has been reported that genes flanked by MAR elements result in tissue independent gene expression and enhanced transcriptional activity. Plasmid DNA pDPG310 was constructed by performing a 3-way ligation among the following DNAs:



- 1) 2.9 kb NotI-KpnI fragment from pBluescript II SK(-),
- 2) 2.95 kb KpnI-PstI MAR fragment from pUC19 B1-X1, and
- 3) 2.1 kb PstI-NotI *bar*-containing fragment from pDPG294.

5 Plasmid pDPG310 contains three SacI sites, one in each of the above DNA fragments. A second MAR element was inserted into the SacI site at the end of the pBluescript II SK(-) multiple cloning region. The resulting plasmid has a unique NotI site, into which future traits of interest could be cloned.

10 In regard to vectors encoding protease inhibitors, plasmid DNA, pRJ15, containing the genomic DNA sequence for the potato *pinII* gene, was obtained from Clarence Ryan (Washington State University) and renamed pDPG288. Another plasmid DNA, pT2-47, containing the cDNA sequence for the tomato *pinII* gene, was obtained from Clarence Ryan and renamed pDPG289. The potato *pinII* gene in  
15 pDPG288 is flanked by a 35S promoter and a Tr7 3' end.

Tandem vectors were constructed containing a *bar* expression unit and a potato or tomato *pinII* expression unit in either convergent or colinear orientation. In each construct, the *bar* and *pinII* expression units were contained on a HindIII  
20 fragment that also contains a 1.9 kb fragment of *Adh1* but lacks the Amp<sup>R</sup> gene and the plasmid origin of replication. The 1.9 kb *Adh1* region provides a locus for recombination into the plant genome without disruption of either the *bar* or *pinII* expression units. These HindIII fragments are available for bombardment experiments as either linear, circular, or supercoiled DNAs.

25 To construct a plant expression vector containing the potato *pinII* terminator, the potato *pinII* terminator was cloned into pBluescript II SK(-) via a three-part ligation. To obtain the *pinII* terminator, a 3.7 kb PstI fragment was first removed from pDPG288 and subsequently digested with RsaI/PstI to yield the 930 bp *pinII*  
30 terminator. The pBluescript II vector was digested with Scal/PstI and Scal/SmaI in

two separate reactions; the appropriate two plasmid fragments were gel-purified. These fragments and the *pinII* terminator were ligated to give plasmid pDPG331.

In order to construct pDPG320, a *pinII* expression vector containing the 35S promoter-*AdhI* intronI-*pinII* (with intron and terminator)-Tr7 terminator vector, the following protocol was used. pDPG309 was cut with BglII/EcoRI and the vector fragment was isolated. pDPG157 was cut with PstI/EcoRI and the 500 bp Tr7 fragment was isolated. To isolate the *AdhI* intron 3'-end, pDPG309 was cut with BglII/XbaI. Finally, pDPG288 was restricted with XbaI/PstI and the potato *pinII* gene (with intron and terminator) was isolated. After purification, the four fragments were ligated together and transformed into competent DH5 $\alpha$  cells. Miniprep analysis identified the correct clone.

A further plant expression vector that contains the potato *pinII* terminator is pDPG343. This plasmid contains the 420-bp 35S promoter, maize *Adh1* intron I, a multiple cloning region, and the potato *pinII* terminator. pDPG343 was constructed by way of a three-part ligation of the following DNAs:

- 1) 3330 bp EcoRI-PstI fragment of pDPG309, containing the pBluescript SK(-) and the 420 bp 35S promoter,
- 2) 580 bp PstI-XbaI fragment of pDPG309, containing the maize *Adh1* intron 1, and
- 3) 960 bp XbaI-EcoRI fragment of pDPG331, containing the potato *pinII* terminator.

Plasmid pDPG343 contains the following restriction sites between the *Adh1* intron I and the potato *pinII* terminator:

BamHI - Sall - XbaI - SpeI - BamHI

Of the above sites, *SpeI* is the only unique one in pDPG343. While *BamHI* can readily be used for one-step cloning of trait genes, use of *Sall* or *XbaI* will require either three-part (or greater) ligations, or multiple cloning steps.

5           The DNA fragment encoding the firefly luciferase protein was inserted into a pUC18-based vector containing the 35S promoter from Cauliflower Mosaic Virus, the first intron from the maize *Adh1* gene, and the transcript 7' 3' end from *Agrobacterium tumefaciens* (provided in the plasmid pCEV1 from Calgene, Inc., Davis, CA). This luciferase expression vector is referred to as pDPG215, and  
10           contains the same regulatory elements as the *bar*-expression vector pDPG165.

Two additional luciferase vectors were created, both utilizing intron VI from *Adh1* (derived from vector pDPG273) fused to firefly luciferase (obtained from vector pDPG215). These elements were inserted into either the pDPG282 (4 OCS  
15           inverted-35S) to create pDPG350, or into the pDPG283 (4 OCS-35S) backbone to create pDPG351 (*bar* was excised as a *BamHI/NheI* fragment and the intron plus luciferase gene inserted). The 4 OCS-35S promoter has been shown with the *uidA* gene to give very high levels of transient expression.

20           Replication-competent viral vectors are also contemplated for use in maize transformation. Two wheat dwarf virus (WDV) "shuttle" vectors were obtained from J. Messing (Rutgers). These vectors, pWI-11 (pDPG386) and pWI-GUS (pDPG387) (Figure 1 T,U), are capable of autonomous replication in cells derived from electroporated maize endosperm protoplasts (Ugaki et al., 1991). Both of  
25           these vectors encode a viral replicase and contain viral and *E. coli* origins of replication. In both of these vectors, the viral coat protein coding sequence has been replaced by the *neo* gene. pDPG387 (pWI-GUS) was created by insertion of a GUS expression cassette (35S-GUS-35S 3') into the *BamHI* site of pDPG386 (pWI-11).

The expression and integration of marker genes introduced into maize cells on a replicating vector was examined using pDPG387 (pWI-GUS) and BMS cells. Six filters of BMS cells were bombarded with pDPG387/pDPG165 (35S-*bar*-Tr7) and as a control, six filters were bombarded with pDPG128

5 (35S-*neo*-Tr7)/pDPG165. Tissue from three filters of each cotransformation was selected on bialaphos (1 mg/l) and tissue from the remaining three filters of each cotransformation was selected on kanamycin (100 mg/l). Selection of kanamycin-resistant colonies from tissue bombarded with pDPG387 was very inefficient (4 colonies) as compared to the control treatment in which cells were  
10 bombarded with pDPG128/pDPG165 (364 colonies). It also appears that bialaphos selection was less efficient for cells bombarded with pDPG387/pDPG165 (59 colonies) than it was for the control in which cells were bombarded with pDPG128/pDPG165 (274 colonies).

15 There are several potential reasons for the low selection efficiencies in bombardments using pDPG387. The *neo* gene carried by pDPG387 is driven by the native WDV coat protein promoter; this promoter may not be strong enough to confer kanamycin resistance. Alternatively, the relatively low number of bialaphos-resistant colonies recovered from cells bombarded with  
20 pDPG387/pDPG165, infers some sort of negative impact of pDPG387 on the ultimate selection for expression of a marker gene on a separate, non-replicating vector. The nature of this negative impact, and its relationship to the low yield of kanamycin-resistant colonies, is currently being investigated.

25 WDV-*bar* vectors were constructed to help address the question of the effect of promoter strength on selection as well as to provide replicating vectors for eventual use with embryogenic cultures. The 35S-*bar*-Tr7 expression cassette was isolated from pDPG165 as an *EcoRI*/*HindIII* fragment and protruding ends were filled in with T4 DNA polymerase. This fragment was ligated into pDPG386  
30 (pWI-11) that had been linearized with *BamHI*, filled in with T4 DNA polymerase,

and alkaline phosphatase treated. Vectors containing both orientations of the insert were generated and designated pDPG388 and pDPG389 (Figure 1 V,W).

A gene encoding the enzyme EPSPS was cloned from *Zea mays*. Two mutations were introduced into the amino acid sequence of EPSPS to confer glyphosate resistance, i.e., a substitution of isoleucine for threonine at amino acid position 102 and a substitution of serine for proline at amino acid position 106. Seven plant expression vectors were constructed using the promoterless mutant maize EPSPS expression vector received from Rhone Poulenc (pDPG425). The mutant EPSPS gene in this vector encodes an enzyme with amino acid changes at positions 102 (threonine to isoleucine) and 106 (proline to serine). Seven promoters ( $\pm$  introns) were used in vector constructions using the mutant maize EPSPS gene. A description of the construction of these vectors is presented below.

Four vectors (pDPG434, 436, 441, and 443) were constructed by cloning four promoters into *Sma*I-linearized pDPG425. Linearized vectors were treated with calf alkaline phosphatase to prevent recircularization prior to ligation. The rice actin promoter and intron were isolated as a 1.5 kb *Hind*III fragment from pDPG421 (pDM302; Cao et al., Plant Cell Rep (1992) 11:586-591). The 35S/adh1 intron I promoter was isolated from pDPG208 as a 0.9 kb *Hind*III fragment. The 2X *Arabidopsis* histone promoter was isolated as a 1.4 kb *Eco*RI/*Hind*III fragment from pDPG404. The 2X 35S/*Arabidopsis* histone promoter was isolated as a 1.8 kb *Eco*RI/*Hind*III fragment from pDPG405. The above mentioned promoter fragments were T4 DNA polymerase-treated to create blunt ends prior to ligation into *Sma*I linearized pDPG425 (Figure 1(Z)). To create pDPG447, a 2.1 *Eco*RI/*Nco*I  $\alpha$ -tubulin/adh1 intron I fragment was isolated from pDPG407 and ligated into *Eco*RI/*Nco*I digested pDPG425 (Figure 1(Z)). The adh1 promoter and intron I were isolated from pDPG172 (Figure 1(Y), a derivative of pDPG140 (Figure 1 (X), as a 1.8 kb *Eco*RI fragment and ligated into *Eco*RI digested, calf alkaline phosphatase-

treated pDPG425 (Figure 1(Z)). The resulting plasmid was designated pDPG465. The 2X OCS promoter and adh1 intron VI were isolated from pDPG354 as a 0.6 kb *SacI/NcoI* fragment and ligated into *SacI/NcoI* digested pDPG425 (Figure 1(Z)). This plasmids was designated pDPG467. A list of all of the mutant maize EPSPS vectors that were constructed is shown in Table 4.

Table 4. List of mutant maize EPSPS plant expression vectors constructed using pDPG425, and various promoters.

DEKALB Plasmid Designation	Plant Expression Cassette
pDPG434	actin - EPSPS - nos
pDPG436	35S/adh1 intron I - EPSPS - nos
pDPG441	2X <i>Arabidopsis</i> histone - EPSPS - nos
pDPG443	2X 35S/ <i>Arabidopsis</i> histone - EPSPS - nos
pDPG447	$\alpha$ -tubulin/adh1 intron I - EPSPS - nos
pDPG465	2X OCS/adh1 intron VI - EPSPS - nos
pDPG467	adh1 promoter/adh1 intron I - EPSPS - nos

Several vectors were constructed containing genes which may increase stress resistance in transgenic plants, including the *mtlD* gene from *E. coli* and the HVA-1 gene from barley. The mannitol operon was originally cloned and characterized by Lee and Saier, 1983 . The *mtlD* gene has been shown to confer stress resistance on transgenic tobacco plants (Tarczynski, M.C. et al., 1993). A plasmid designated pCD7.5, containing the *mtlD* gene from this operon (encoding mannitol-1-phosphate dehydrogenase) was obtained from M. Müller, University of

Freiburg. The structural gene was isolated as a 1500 bp fragment after digestion of pCD7.5 with NsiI and PstI, and was ligated into a pUC18-based vector containing the 35S promoter from Cauliflower Mosaic Virus, the first intron from the maize Adh1 gene, and the transcript 7' 3' end from *Agrobacterium tumefaciens*.

5 The backbone and regulatory elements were prepared for this construction by removing the luciferase structural gene from pDPG215 (35s-Adh1<sub>1</sub>-*luc*-Tr7 3'; further described in this document), and then religating with an oligonucleotide that created a unique NsiI site between the intron and Tr7 element (this intermediate vector was designated pDPG431). pDPG431 was then linearized using NsiI and  
10 the *mtlD* gene was inserted. The final vector was designated pDPG451 (Figure 1 (BB)).

A second expression vector for the *mtlD* gene was created by removing the  
15 *bar* gene from pDPG182 using SmaI. After blunting the ends of the *mtlD* gene, it was ligated into the pUC-based vector; between the maize Adh1 promoter/Adh1<sub>1</sub> intron and the transcript 7' 3' end from *Agrobacterium tumefaciens* (provided in pCEV5 from Calgene, Inc., Davis, CA). This plasmid vector was designated pDPG469.

20 A gene isolated from barley that encodes a Late Embryogenic Abundant protein (Dure, L., et al., 1989). (HVa-1) was obtained from Dr. H.D. Ho (Washington University), isolated as an NciI/SphI fragment and cloned into the polylinker of pCDNA II (Invitrogen, Inc). It was then reisolated as a BamHI/NsiI  
25 fragment and cloned into the polylinker site of pDPG431 (see above). The result was the pUC-based vector with the following expression unit, 35s-Adh1<sub>1</sub>-HVa1-Tr7 3' (designated pDPG474).

Plasmid constructs designed for increasing the level of lysine in the plant  
30 were designed to place a *dapA* polypeptide-coding sequence, modified to contain a

sequence corresponding to one of two maize plastid transit peptide sequences, under the control of various plant promoter elements. These constructs include the widely-used CaMV 35S promoter, maize endosperm-specific promoters from genes encoding either a 27 kD (Z27) or a 10 kD (Z10) zein storage protein, and an embryo-specific promoter from the maize Globulin1 gene (Glb1), which encodes an abundant embryo storage protein. The transit peptide sequences used here correspond to those present in genes encoding either a maize rubisco small subunit polypeptide (MZTP) or the native maize DHDPs polypeptide (DSTP). Features of these constructs, along with their lab designations, are as follows:

expression

construct    promoter/transit peptide/coding seq./3' regulatory sequence

pDPG 334	Z10/MZTP/ <i>dapA</i> /nos
335	Z27/DSTP/ <i>dapA</i> /35S
371	35S/MZTP/ <i>dapA</i> /nos
372	35S/DSTP/ <i>dapA</i> /nos
418	Glb1/DSTP/ <i>dapA</i> /Glb1


Construction of these plasmids was performed as follows:

pDPG371: In this construct, the synthetic pea chloroplast transit peptide encoding sequence described in U.S. Patent Application Serial No. 07/204,388 was replaced with a synthetic sequence corresponding to that encoding a maize chloroplast transit peptide (from a rubisco small subunit sequence; GenBank Accession Y00322). Eight oligonucleotides were synthesized in order to reconstruct the maize ssu transit peptide sequence by the same strategy described for synthesis of the pea chloroplast transit peptide sequence (in U.S. Patent Application Serial No. 07/204,388). Sequences of these oligonucleotides, in the



the final context of the synthetic gene fragment, are as follows:

HindIII MZTP46 MZTP49  
AGCTTGCAGCGAGTACATACATACTAGGCAGCCAGGCAGCC**ATGGCGCCACC**  
5 ACGTCGCTCATGTATGTATGATCCGTCGGTCCGTCGGTACCGCGGGTGG  
MZTP25 MZTP45  
MZTP51  
GTGATGATGGCCTCGTCGGCCACCGCCGTCGCTCCGTTCCAGGGGCTCAAGTCC  
10 CACTACTACCGGAGCAGCCGGTGGCGGCAGCGAGGCAAGGTCCCCGAGTTCAGG  
MZTP53  
MZTP39  
ACCGCCAGCCTCCCCGTCGCCCCGCCGGTCCTCCAGAAGCCTCGGCAACGTCAGC  
TGGCGGTTCGGAGGGGCAGCGGGCGGCCAGGAGGTCTTCGGAGCCGTTGCAGTCG  
15 MZTP54  
SphI  
AACGGCGGAAGGATCCGGTGCATG  
TTGCCGCCTTCCTAGGCCAC

Ans.   
20 B' The sequence is identical to that of the rubisco ssu gene described in  
GenBank Y00322, except for the introduction of a HindIII-compatible sequence  
generated by the addition of AGCTT to the 5' end of MZTP46 and an A residue to  
the 3' end of MZTP25. The ATG initiation codon is indicated in bold type, as is the  
TGC cysteine codon corresponding to the carboxyterminal residue of the native  
25 maize ssu transit peptide. This corresponds to a 47 amino acid transit peptide  
sequence, from the initiating methionine to the carboxyterminal cysteine.

30 Using the same strategy as described for synthesis and reconstruction of the  
pea transit peptide sequence, equimolar amounts of oligonucleotides MZTP49, 51,  
39, 25, 45, and 53 were phosphorylated at their 5' ends in a polynucleotide kinase  
reaction; these were then combined with equimolar amounts of MZTP46 and  
MZTP54. This mixture was then added to a ligation reaction mixture containing  
the pGem3 vector (Promega Biotec, Madison, WI) which had previously been

cleaved with the restriction enzymes HindIII and SphI to yield plasmid 9106. To fuse the ssu transit peptide sequence (MZTP) to the *dapA* polypeptide coding sequence, a 1170 bp SphI/EcoRI fragment, corresponding to the *dapA*/nos 3' cassette present in plasmid pDAP4201 (in U.S. Patent Application Serial No. 07/204,388), was ligated into plasmid 9106 that had been cleaved with SphI and EcoRI to yield pDAP9284. To place this novel construct under control of the plant 35S promoter, a 1300 bp HindIII fragment from pDAP9284, containing the MZTP/*dapA*/nos3' cassette, was ligated into the HindIII site of plasmid 35-227 (U.S. Patent Application Serial No. 07/204,388), which contains the 35S promoter, to yield plasmid 9305. To facilitate use of the 35S/MZTP/*dapA*/nos3' cassette in future manipulations, the entire cassette was isolated as a 1790 bp ClaI/SmaI fragment from plasmid 9305 and inserted into the commercial cloning vector pSP73 (Promega) which had been cleaved with ClaI and SmaI. This final construct is designated pDPG371.

pDPG335: In this construct the synthetic MZTP ssu transit peptide sequence was replaced with DNA encoding the transit peptide sequence from the native maize DHDPS enzyme. The plasmid pMDS-1, containing a cDNA clone corresponding to maize DHDPS (Frisch et al. Mol. Gen. Genet. 228:287-293, 1991), was a gift from B. Gengenbach, University of Minnesota, Minneapolis. The EcoRI fragment containing the full-length maize DHDPS cDNA was inserted into EcoRI-cleaved pSP73 to yield pMDS73A; this step was performed to facilitate subsequent cloning steps.. A 215 bp ClaI/XbaI fragment from pMDS73A containing the transit peptide coding region was inserted into the ClaI and XbaI sites of plasmid p35-227, thereby fusing the transit peptide sequence to the 35S promoter sequence contained in p35-227 and yielding the plasmid pPol35SDTP. The 35S/DSTP cassette was subsequently fused to the *dapA* coding sequence. This was performed by cloning the cassette into the plasmid pHDA73, which was constructed as follows: The 2728 bp hygromycin phosphotransferase cassette from plasmid pHygI1 (U.S. Patent Application Serial No. 07/204,388) was cloned

as an EcoRI/HindIII fragment into the EcoRI and HindIII sites of pSP73, to yield pHyg73. This plasmid was then cleaved with BglII and ClaI, and the 35S/MZTP/*dapA*/nos cassette obtained as an 1800bp BglII/ClaI fragment from pDPG371 was inserted to yield pHDA73. This latter plasmid was then cleaved at the SphI site, at which point the MZTP sequence joins the *dapA* sequence. The SphI 3' overhang was filled in by using Klenow fragment to produce a blunt end, and the resultant linear plasmid was subsequently cleaved with BglII, removing the 35S/MZTP portion from pHDA73. The 35S/DSTP cassette was isolated from pPol35SDTP by cleavage with XbaI, followed by digestion with mung bean nuclease to remove the resultant 5' overhang. This treatment was followed by BamHI cleavage, which yielded a 674 bp fragment that was inserted into the cleaved pHDA73 plasmid in place of the 35S/MZTP sequence. This novel plasmid, containing the cassette 35S/DSTP/*dapA*/nos, is designated pHDT. A 1095 bp EcoRI/BamHI fragment, containing the DSTP/*dapA* region, was cloned into the EcoRI and BamHI sites of the commercial vector pUC119 (BRL) to yield pDSTP119. Nucleotide sequence analysis of this latter clone revealed a 2 bp deletion, apparently caused by the cloning process, at the junction of the DSTP and *dapA* sequences. This mutation was corrected in such a way as to restore the original reading frame and to introduce an additional MaeII restriction enzyme site as follows:

	DSTP	<i>dapA</i>
target sequence:	ATC <u>ACT</u>	TTC ACG GGA
<u>CT</u> deleted in pDSTP119	ile thr	phe thr gly
modify to:	ATC ACG	TTC ACG GCA

This manipulation leaves the desired reading frame intact and introduces the ACGT MaeII site at the junction between the DSTP and *dapA* sequences. Mutagenesis was accomplished with the reagents supplied in the BioRad Mutagene kit by using the oligonucleotide:

5' CCTTGGCAGCCATCACGTTACGGGAAGTATTGTC 3'

The resultant plasmid, designated pMae2-1, was cleaved with EcoRI, treated with Klenow polymerase to generate blunt ends, then cleaved with BamHI to yield an 1100bp fragment consisting of the DSTP/*dapA* cassette. This fragment was cloned into the Sma and BamHI sites of pZ27Z10 (in U.S. Patent Application Serial No. 07/636,089), replacing the Z10 coding region and placing the DSTP/*dapA* sequence under control of the Z27 promoter and the 35S 3' regulatory region. This final construct is designated pDPG335.

pDPG372: To place the DSTP/*dapA* cassette under control of the 35S promoter, a 1221 bp Scal/EcoRI fragment from pHOTP, containing pSP73 vector sequences and the 35S promoter, was inserted into the Scal and EcoRI sites of pMae2-1 to yield p35MDAP. To join the nos 3' regulatory region to the 35S/DSTP/*dapA* cassette, an 800bp fragment of p35MDAP from the NdeI site just upstream of the 35S promoter to an NheI site internal to the *dapA* sequence was inserted into the NdeI and NheI sites of pDPG371, effectively replacing the 35S/MZTP cassette with the 35S/DSTP sequence. The resultant plasmid, containing the 35S/DSTP/*dapA*/nos 3' cassette, is designated p35DSD. Subsequent nucleotide sequence analysis of 35DSD revealed the presence of a cloning artifact that introduced an ATG translation initiation codon 13 codons upstream of the authentic DSTP initiation codon. This problem was corrected by substituting this region of p35DSD with the corresponding region from pDPG335, as follows: a 525 bp fragment from pDPG335, extending from a KpnI site at the 3' end of the 35S promoter to a BstEII site internal to the *dapA* sequence, was inserted into KpnI/BstEII-cleaved p35DSD to yield pDPG372, which contains a functional 35S/DSTP/*dapA*/nos cassette.

pDPG334: In this construct, the MZTP/*dapA*/nos cassette was placed under control of the Z10 promoter region as follows: a 1137 bp HindIII/NcoI fragment

from pG10B-H3 (Kiriwara et al, Gene 71:359-370, 1988), consisting of the Z10 promoter, was inserted into HindIII/NcoI cleaved pDAP9284, yielding pDPG334 which consists of the Z10/MZTP/*dapA*/nos cassette.

5     \_\_\_\_\_ pDPG418: In this construct, the DSTP/*dapA* cassette was placed under control of the 5' and 3' regulatory regions of the maize Globulin1 (Glb1) gene (Belanger and Kriz Genetics 129:863-872, 1991) as follows: a 1050 bp KpnI/PstI fragment from pDPG335, consisting of the DSTP/*dapA* cassette, was inserted into the KpnI/PstI sites of the Glb1 expression vector pGEMSV3 (GenBank Accession  
10    No. L22295) to yield pDPG418.

Features of introduced genes used for selection of transformed cells are described above. Specific plasmid constructs used in these experiments are as follows:

15

selection

construct     Selectable/screenable marker

	pDPG 165	bar
20	231	bar , gus
	283	bar
	355	gus
	363	bar
	366	hpt
25	367	hpt

Constructs pDPG 165, 231, 283, and 363 are as described above. Constructs pDPG355 and 367 are described in Walters et al., 1992 as pBII221 and pHygI1, respectively. Construct pDPG366 was made by transferring the hygromycin  
30    expression cassette (35S/Adh11/hpt/nos) from pDPG367 as an EcoRI/HindIII

fragment into EcoRI/HindIII cleaved pSP73.

Three plasmids containing the gene encoding the 10 kD zein protein were constructed. The plasmid pDPG375 is a 7 kb pUC119 plasmid containing a 3.9 kb  
5 *HindIII* fragment of a genomic clone encoding the 10 kD zein (Kiriwara et al., 1988). This gene is under control of the native promoter, and contains the native 3' sequence. The plasmid pDPG373 is a pUC119-based plasmid containing a *HindIII*-*RsaI* Z4 (22 kD zein) promoter fragment and an *NcoI*-*EcoRI* fragment with the 10 kD zein coding and 3' sequences, and pDPG338 is a pUC119-based plasmid  
10 containing the 1.1 kb 27 kD zein promoter, 2.3 kb of the 10 kD zein coding and 3' sequences, and a cauliflower mosaic virus (CaMV) 35S poly(A) sequence. These plasmids are hereafter designated as the methionine constructs. Selectable marker genes used were hygromycin phosphotransferase (HPT, pHYG11 also known as pDPG367; 35S promoter::Adh1 intron::HPT coding sequence:: nos  
15 poly(A)sequence; Walters et al., 1992) and *bar*, the gene conferring resistance to the herbicide Basta (35S::bar::Tr7, pDPG165, described earlier in this CIP) or pDPG363 (35S::Adh1::bar::nos). The plasmid pDPG363 was constructed by inserting a 0.5 kb *SmaI* fragment with *Bam*HI linkers containing the *bar* gene into pHYG73, replacing the HPT gene. The plasmid pHYG73 was constructed by  
20 insertion of a 2.1 kb *EcoRI*-*HindIII* fragment containing the HPT coding sequence from pDPG367 (cited above) into pSP73 (Promega). The screenable marker gene of pBI221, which encodes  $\beta$ -glucuronidase (GUS) was also used (35S::Adh1::GUS::nos). The plasmid pBI221 was constructed by adding a 0.75 kb fragment containing the Adh1 intron between the 35S CaMV promoter and the  
25 GUS coding sequence of pBI221 (Clontech; pBI221 is pBI121 in pUC19 rather than pBIN19; Jefferson et al., 1987).

The plasmid pDPG380 contains the entire *BalI*-*EcoRI* 711 bp coding sequence of the gene encoding the 19 kD A20 zein and the 0.5 kb 5' sequence  
30 encoding the A20 preprotein (reconstructed independently from the coding

sequence by PCR) in antisense orientation, with 1137 bp of the 10 kD zein promoter and 250 bp of nos 3' sequence. The plasmid pDPG340 is a pUC119-based plasmid containing 1137 bp of Z10 promoter sequence, a 980 bp *Xba*I- *Sac*I fragment with the entire Z4 coding sequence in antisense orientation, and 250 bp of nos 3' sequence. These genes are hereafter referred to as antisense constructs. For transformation experiments, pDPG165 (35S::bar::Tr7, described elsewhere in this CIP), pDPG363 (35S::Adh1::bar::nos, also described elsewhere in this CIP) or pDPG367 (35S::Adh1::HPT::nos, described elsewhere) were used as selectable marker genes, and were cobombarded with the antisense constructs.

The vector pDPG354 contains an expression cassette for producing Bt endotoxin in maize (see Figure 1 (CC) for map). It was constructed to contain the following DNA:

- (i) A promoter, consisting of two *ocs* enhancers (J.G. Ellis et al., 1987 ) placed in the reverse orientation and located upstream of the TATA box derived from the cauliflower mosaic virus (CaMV) 35S promoter (*Eco* RV site to the transcription start site; H.Guilley et al.,1982 ; R.Kay et al.1987 ), located upstream from:
- (ii) an intron (intron VI) derived from the maize Adh1 gene (Callis, J., Fromm, M., Walbot, V. ,1987), a 423bp *Acc*I-*Msp*I fragment from a genomic clone of the Adh1 gene) that was located upstream from:
- (iii) a synthetic Bt gene on a *Nco* I to *Kpn* I fragment of DNA coding for the toxin portion of the endotoxin protein produced by *Bacillus thuringiensis* subsp.kurstaki strain HD73 (M.J. Adang et al. 1985 ). This gene was synthesized and assembled using standard techniques to contain codons that are more preferred for translation in maize cells. A translation stop codon

was introduced after the 613th codon to terminate the translation and allow synthesis of a Bt endotoxin protein consisting of the first 613 amino acids (including the f-met) of the Bt protein (see Figure 12 for sequence of gene).

- 5 (iv) The DNA coding for the Bt protein was followed by a 930bp *RsaI-PstI* segment of DNA derived from the potato proteinase II inhibitor gene (G.An et al., 1989).

10 This expression cassette was inserted into the *E.coli* plasmid pBluescript SK- (Stratagene, Inc.) and can be made available from the ATCC.

The vector pDPG344 was designed to mediate the expression of the tomato protease inhibitor II (*pin*) gene in maize and was constructed to contain the following DNA (see Figure 1 (DD):

15

- (i) 420bp of DNA derived from the 35S promoter from CaMV located upstream of:
- (ii) 550bp derived from the intron1 of the maize *Adhl* gene (Callis, J., Fromm, M.E., Walbot, V., 1987. ) located immediately upstream from :
- 20 (iii) 470bp of DNA coding for the cDNA of the tomato protease inhibitor II gene (Graham et al., 1985), followed by:
- (iii) 930bp of DNA derived from the 3' region of the potato protease inhibitor II gene (derived from pRJ13; Johnson et al., 1989);

25

This cassette was assembled on the *E.coli* plasmid pBluescript SK(-) (Stratagene Inc.) and can be made available from the ATCC.

The plasmid vector pDPG337 (also known as pLK487) consists of an *E.coli* replicon (pBS+;Stratagene Inc.) containing the following DNA (see Figure 1 (EE)):

30



1. a segment of DNA containing the promoter for the 35S transcript derived from CaMV, fused at the location of initiation of transcription of the 35S transcript to:
- 5  
B4 → 2. a segment of DNA (5' ATC TGG CAG CAG AAA AAC AAG TAG TTG AGA ACT AAG AAG AAG AAA 3')<sup>B4</sup> derived from the untranslated 5' leader sequence to the small subunit of the ribulose biscoxylase gene of soybean (S.L.Berry-Lowe et al.,1982) which is joined at its 3' terminus to:
- 10 3. a synthetic Bt gene coding for the endotoxin from *Bacillus thuringiensis* subsp.*kurstaki* strain HD73 (see construction of pDPG 354 above).
- 15 4. The Bt gene was followed by a segment of DNA derived from the 3' of "transcript 7" gene from *Agrobacterium tumefasciens* (P. Dhaese et al.,1983,).

Samples of *E.coli* containing pDPG337 can be made available through the ATCC.

#### Example 7: Intracellular Targeting of the *bar* Gene

20 As mentioned above, the *bar* gene codes for an enzyme, PAT, that inactivates the herbicide phosphinothricin. Phosphinothricin is an inhibitor of both cytoplasmic and chloroplast glutamine synthetases. Current expression vectors target PAT to the cell cytoplasm. To determine if there is an advantage to also  
25 targeting PAT to the chloroplast, a transit:*bar* chimeric gene was constructed. A sequence encoding the *rbcS* transit peptide and part of the *rbcS* mature polypeptide, present on a 300 bp XbaI-BamHI fragment from pDPG226, was cloned adjacent to the *bar* sequence in pDPG165, to produce pDPG287. This resulted in  
30 an in-frame fusion of the two protein coding regions, with the intervening sequence coding for the following amino acids:

*rbcS-Pro-Arg-Gly-Ser-Thr-bar*

Protoplasts were electroporated with pDPG287 and assayed for PAT activity. Using proteinase treatment and protection studies, it was then determined that the PAT enzyme is sequestered within an organelle, particularly the plastid.

#### F. Preferred Methods of Delivering DNA to Cells

Preferred DNA delivery systems do not require protoplast isolation or use of *Agrobacterium* DNA. There are several potential cellular targets for DNA delivery to produce fertile transgenic plants: pollen, microspores, meristems, immature embryos and cultured embryogenic cells are but a few examples. Germline transformation in maize has not been previously reported.

One of the newly emerging techniques for the introduction of exogenous DNA constructs into plant cells involves the use of microprojectile bombardment. The details of this technique and its use to introduce exogenous DNA into various plant cells are discussed in Klein, 1989, Wang, *et al.*, 1988 and Christou, *et al.*, 1988. One method of determining the efficiency of DNA delivery into the cells via microprojectile bombardment employs detection of transient expression of the enzyme  $\beta$ -glucuronidase (GUS) in bombarded cells. For this method, plant cells are bombarded with a DNA construct which directs the synthesis of the GUS enzyme.

Apparati are available which perform microprojectile bombardment. A commercially available source is an apparatus made by Biolistics, Inc. (now DuPont), but other microprojectile or acceleration methods are within the scope of this invention. Of course, other "gene guns" may be used to introduce DNA into cells.

Several modifications of the microprojectile bombardment method were made by the inventors. For example, stainless steel mesh screens were introduced below the stop plate of the bombardment apparatus, i.e., between the gun and the cells. Furthermore, modifications to existing techniques were developed by the inventors for precipitating DNA onto the microprojectiles.

Another newly emerging technique for the introduction of DNA into plant cells is electroporation of intact cells. The details of this technique are described in Krzyzek and Laursen (PCT publication WO 92/12250). Similar to particle bombardment, the efficiency of DNA delivery into cells by electroporation can be determined by using the  $\beta$ -glucuronidase gene. The method of electroporation of intact cells and by extension intact tissues, e.g., immature embryos, were developed by Krzyzek and Laursen and represent improvements over published procedures. Generation of fertile plants using these techniques were described by Spencer et al. (Spencer, T.M., Laursen, C.M., Krzyzek, R.A., Anderson, P.C., and Flick, C.E., 1993, Transgenic maize by electroporation of pectolyase-treated suspension culture cells. Proceedings of the NATO Advanced Study Institute on Plant Molecular Biology, Molecular-Genetic Analysis of Plant Metabolism and Development, In Press.) and Laursen et al. (Laursen, C.M., Krzyzek, R.A., Flick, C.E., Anderson, P.C. and Spencer, T.M. Transformation of maize by electroporation of suspension culture cells. Submitted to Plant Molecular Biology.)

Other methods may also be used for introduction of DNA into plants cells, e.g., agitation of cells with DNA and silicon carbide fibers.

**Example 8: Microprojectile Bombardment--SC82, SC94 and SC716**

For bombardment, friable, embryogenic Type-II callus (Armstrong & Green, 1985) was initiated from immature embryos essentially as set forth above in Examples 1 and 2. The callus was initiated and maintained on N6 medium (Chu *et al.*, 1975) containing 2 mg/l glycine, 2.9 g/l L-proline, 100 mg/l casein hydrolysate, 13.2 mg/l dicamba or 1 mg/l 2,4-D, 20 g/l sucrose, pH 5.8, solidified with 2 g/l Gelgro (ICN Biochemicals). Suspension cultures initiated from these callus cultures were used for bombardment.

In the case of SC82, suspension culture SC82 was initiated from Type-II callus maintained in culture for 3 months. SC82 cells (see Example 2) were grown in liquid medium for approximately 4 months prior to bombardment (see Table 5, experiments #1 and #2). SC82 cells were also cryopreserved 5 months after suspension culture initiation, stored frozen for 5 months, thawed and used for bombardment (experiment #6).

In the case of suspension culture SC716 (see Example 1), it was initiated from Type-II callus maintained 5 months in culture. SC716 cells were cultured in liquid medium for 5 months, cryopreserved for 8 months, thawed, and used two months later in bombardment experiments #4 and #5. SC94 was initiated from 10 month old Type-II callus; and cultured in liquid medium for 5 months prior to bombardment (experiment #3).

Prior to bombardment, recently subcultured suspension culture cells were sieved through 1000  $\mu$ m stainless steel mesh. From the fraction of cell clusters passing through the sieve, approximately 0.5 ml packed cell volume (PCV) was pipetted onto 5 cm filters (Whatman #4) and vacuum-filtered in a Buchner funnel. The filters were transferred to petri dishes containing three 7 cm filters (Whatman #4) moistened with 2.5 ml suspension culture medium.

The dish containing the filters with the suspension cells was positioned 6 cm below the lexan plate used to stop the nylon macroprojectile. With respect to the DNA, when more than a single plasmid was used, plasmid DNA was precipitated in an equimolar ratio onto tungsten particles (average diameter approximately 1.2  $\mu\text{m}$ , GTE Sylvania) using a modification of the protocol described by Klein, *et al.* (1987). In the modified procedure, tungsten was incubated in ethanol at 65°C for 12 hours prior to being used for precipitation. The precipitation mixture included 1.25 mg tungsten particles, 25  $\mu\text{g}$  plasmid DNA, 1.1 M  $\text{CaCl}_2$  and 8.7 mM spermidine in a total volume of 575  $\mu\text{l}$ . After adding the components in the above order, the mixture was vortexed at 4° C for 10 min, centrifuged (500 X G) for 5 min and 550  $\mu\text{l}$  of supernatant was decanted. From the remaining 25  $\mu\text{l}$  of suspension, 1  $\mu\text{l}$  aliquots were pipetted onto the macroprojectile for bombardment.

Each plate of suspension cells was bombarded twice at a vacuum of 28 inches Hg. In bombarding the embryogenic suspensions of A188 X B73 and A188 X B84, 100  $\mu\text{m}$  or 1000  $\mu\text{m}$  stainless steel screens were placed about 2.5 cm below the stop plate in order to increase the number of foci while decreasing their size and also to ameliorate injury to the bombarded tissue. After bombardment, the suspension cells and the supporting filter were transferred onto solid medium or the cells were scraped from the filter and resuspended in liquid culture medium.

Cells from embryogenic suspension cultures of maize were bombarded with the *bar*-containing plasmid pDPG165 alone or in combination with a plasmid encoding GUS, pDPG208 (Fig. 1). In experiments in which a GUS plasmid was included, two of the filters containing bombarded cells were histochemically stained 48h post-bombardment. The total number of foci (clusters of cells) per filter transiently expressing GUS was at least 1000. In two separate studies designed to quantitate transiently expressing cells (using an SC82 (A188 x B73) suspension culture), the mean number of GUS-staining foci per filter was 1472 and 2930. The number of cells in individual foci that expressed GUS averaged 2-3

(range 1-10). Although histochemical staining can be used to detect cells transformed with the gene encoding GUS, those cells will no longer grow and divide after staining. For detecting stable transformants and growing them further, e.g., into plants, selective systems compatible with viability are required.

5

#### **Example 9: Microprojectile bombardment: AB12**

10 Cell line AB12 was initiated as described in example 4. The microprojectile bombardment instrument, microprojectiles and stopping plates were obtained from Biolistics (Ithaca, NY). Five clumps of 7- to 12-day-old callus, each approximately 50 mg in wet weight, were arranged in a cross pattern in the center of an empty 60 mm x 15 mm Petri plate. Plates were stored in a closed container with moist paper towels throughout the bombardment process.

15 Plasmids were coated onto M-10 tungsten particles (Biolistics) as described by Klein et al. (1988, 1989) except that 5  $\mu$ g of DNA was used, the DNA precipitation onto the particles was performed at 0 °C and the tubes containing the DNA-coated tungsten particles were stored on ice throughout the bombardment process. When both plasmids were used, each was present in an amount of 2.5  
20  $\mu$ g. Control bombardments contained TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) with no DNA.

25 The sample plate tray was placed 5 cm below the bottom of the stopping plate tray of the microprojectile instrument, with the stopping plate platform in the slot nearest to the barrel. A 3 mm x 3 mm mesh galvanized steel screen was placed over the open dish. The instrument was operated as described by the manufacturer (Biolistics, Inc.), using a gunpowder charge as the motive force. Each plate of tissue was bombarded once.

### Example 10: Microprojectile Bombardment--AT824

Suspension culture AT824 (described in example 3) was subcultured to fresh medium 409 2 days prior to particle bombardment. Cells were plated on solid 409 medium 16-24 hours before bombardment (about 0.5 ml packed cell volume per filter). Tissue was treated with 409 medium containing 200 mOsm sorbitol (medium 431) for 1 hour prior to bombardment.

DNA was introduced into cells using the DuPont Biolistics PDS1000He particle bombardment device.

DNA was precipitated onto gold particles as follows. A stock solution of gold particles was prepared by adding 60 mg of 1 um gold particles to 1000 ul absolute ethanol and incubating for at least 3 hours at room temperature followed by storage at -20C. Twenty to thirty five ul sterile gold particles are centrifuged in a microcentrifuge for 1 min. The supernatant is removed and one ml sterile water is added to the tube, followed by centrifugation at 2000 rpm for 5 minutes. Microprojectile particles are resuspended in 30 ul of DNA solution (30 ug total DNA) containing 10 ug each of the following vectors: pDPG165 (*bar*), pDPG344 (tomato proteinase inhibitor II gene), and pDPG354 (*B. thuringiensis* crystal toxin protein gene). Two hundred twenty microliters sterile water, 250 ul 2.5 M CaCl<sub>2</sub> and 50 ul spermidine are added. The mixture is thoroughly mixed and placed on ice, followed by vortexing at 4C for 10 minutes and centrifugation at 500 rpm for 5 minutes. The supernatant is removed and the pellet resuspended in 600 ul absolute ethanol. Following centrifugation at 500 rpm for 5 minutes the pellet is resuspended in 36 ul of absolute ethanol .

Ten ul of the particle preparation were dispensed on the surface of the flyer disk and the ethanol was allowed to dry completely. Particles were accelerated by a helium blast of approximately 1100 psi. One day following bombardment cells

were transferred to liquid medium 409 (10 ml). Tissue was subcultured twice per week. During the first week there was no selection pressure applied.

#### **Example 11: Further Optimization of Ballistic Transformation**

This example describes the optimization of the ballistic transformation protocol. Both physical and biological parameters for bombardment have been addressed. Physical factors are those that involve manipulating the DNA/microprojectile precipitate or those that affect the flight and velocity of either the macro- or microprojectiles. Biological factors include all steps involved in manipulation of cells immediately after bombardment. The prebombardment culturing conditions, such as osmotic environment, the bombardment parameters, and the plasmid configuration have been adjusted to yield the maximum numbers of stable transformants.

#### **Physical Parameters**

**Gap Distance** The variable nest (macro holder) can be adjusted to vary the distance between the rupture disk and the macroprojectile, i.e., the gap distance. This distance can be varied from 0 to 2 cm. The predicted effects of a shorter gap are an increase of velocity of both the macro- and microprojectiles, an increased shock wave (which leads to tissue splattering and increased tissue trauma), and deeper penetration of microprojectiles. Longer gap distances would have the opposite effects but may increase viability and therefore the total number of recovered stable transformants.

The effect of gap distance was investigated by bombarding the E1 suspension with pDPG208. Plates were shot in triplicate at gaps of 3, 6, 9, and 12 mm. Tissue was assayed for GUS activity and foci were counted. Using a 3 mm gap, GUS foci were the most numerous and well distributed across the filter.



The gas shock wave appeared to be the greatest at this distance as shown by the degree of tissue splattering. Previous experiments performed at this gap size have also shown poor tissue recovery. Gaps of 6 mm and 9 mm showed little to no tissue splattering. GUS foci were well distributed across the filter but were fewer in number than those in the 3 mm samples. Samples bombarded with a gap distance of 12 mm showed nearly equivalent numbers of GUS foci as with sample bombarded at 6 mm and 9 mm but they were located almost exclusively at the center of the filter. No tissue splattering was observed. Based on these observations, it is suggested that bombardments be conducted with a gap distance of 6 to 9 mm.

**Flight Distance** The fixed nest (contained within the variable nest) can be varied between 2 and 2 cm in predetermined increments by the placement of spacer rings to adjust the flight path traversed by the macroprojectile. Short flight paths allow for greater stability of the macroprojectile in flight but reduces the overall velocity of the microprojectiles. Increased stability in flight increases the number of centered GUS foci. Greater flight distances (up to some point) increase velocity but also increases instability in flight.

The effect of the macroprojectile flight path length was investigated using E1 suspension cells. The flight distances tested were 0, 1.0, 1.5, and 2.0 cm. Samples were bombarded with pDPG208 GUS vector and were assayed 48 hours after bombardment for GUS activity. The number of GUS foci was the greatest at a flight path length of 1.0 cm and least at 0 cm. No tissue splattering was observed at 0 cm, very little at 1.0 cm, and greater amount at 1.5 and 2.0 cm. Based on these observations, it is recommended that bombardments be done with a flight path length of 1.0 cm.

**Tissue distance** Placement of tissue within the gun chamber should have significant effects on microprojectile penetration. Increasing the flight path of the

microprojectiles will decrease velocity and trauma associated with the shock wave. A decrease in velocity will also result in shallower penetration of the microprojectiles.

5           **Helium pressure** By manipulation of the type and number of rupture disks, pressure can be varied between 400 and 2000 psi within the gas acceleration tube. Optimum pressure for stable transformation has been determined to be between 1000 and 1200 psi.

## 10    **Biological Parameters**

          Culturing conditions and other factors can influence the physiological state of the target cells and may have profound effects on transformation and integration efficiencies. First, the act of bombardment could stimulate the production of  
15   ethylene which could lead to senescence of the tissue. The addition of antiethylene compounds could increase transformation efficiencies. Second, it is proposed that certain points in the cell cycle may be more appropriate for integration of introduced DNA. Hence synchronization of cell cultures may enhance the frequency of production of transformants. Third, the degree of tissue  
20   hydration may also contribute to the amount of trauma associated with bombardment as well as the ability of the microprojectiles to penetrate cell walls.

          It has also been reported that slightly plasmolyzed yeast cells allow increased transformation efficiencies (Armaleo *et al.*, 1990). It was hypothesized  
25   that the altered osmotic state of the cells helped to reduce trauma associated with the penetration of the microprojectile. Lastly, the growth and cell cycle stage may be important with respect to transformation.

**Osmotic adjustment** It has been suggested that osmotic pre-treatment could  
30   potentially reduce bombardment associated injury as a result of the decreased

turgor pressure of the plasmolyzed cell. Two studies were done in which E1 suspension cells were osmotically adjusted with media supplemented with sorbitol. Cells were plated onto osmotic media 24 hours prior to bombardment. The osmotic values of the media were 200, 400, and 600 mOSM/kg. Samples were  
5 bombarded with either pDPG208 (GUS) or a coprecipitate of pDPG165 (*bar*) and pDPG290 (*Bt*). GUS samples were assayed and foci were counted and plotted. Cells osmotically adjusted at 400 mOSM/kg showed an approximately 25% increase in the number of transient GUS foci. Samples bombarded with *bar/Bt* were selected in liquid (2 mg/l bialaphos) and thin plated on medium containing 3  
10 mg/l bialaphos. Cells treated with 600 mOSM/kg medium grew more slowly than cells treated with media of other osmotic strengths in this study.

A second study investigated the effects of short duration osmotic adjustment at 500 mOSM/kg on both transient GUS expression and stable  
15 transformation. The rationale for the short duration of osmotic adjustment was that cells should be plasmolyzed just before bombardment, using longer time periods of pretreatment may allow the cells to adjust to the osmoticum (i.e. re-establishing turgor). The first control was bombarded (0 min., no new medium) followed by cells pretreated for 45 minutes and 90 minutes with 500 mOSM/kg  
20 medium with either pDPG208 or pDPG165 with pDPG290. Since the pretreatment required media changes (i.e. fresh 500 mOSM/kg media), a set of controls were also washed using fresh medium without the osmoticum. After bombardment the cells were put on to solid medium to recover overnight followed by resuspension in liquid medium. After one week, liquid selection was started using 2 $\mu$ g/ml  
25 bialaphos. Cells were plated on 3 $\mu$ g/ml bialaphos at 0.1 ml PCV eleven days after bombardment. Transient GUS activity was assayed 48 hours after bombardment.

The number of cells transiently expressing GUS increased following subculture into both fresh medium and osmotically adjusted medium. Pretreatment  
30 times of 90 minutes showed higher numbers of GUS expressing foci than shorter

times. Cells incubated in 500 mOSM/kg medium for 90 minutes showed an approximately 3.5 fold increase in transient GUS foci than the control.

**Plasmid configuration** In some instances it will be desirable to deliver DNA to maize cells that does not contain DNA sequences necessary for maintenance of the plasmid vector in the bacterial host, e.g., *E. coli*, such as antibiotic resistance genes, including but not limited to ampicillin, kanamycin, and tetracycline resistance, and prokaryotic origins of DNA replication. In one such experiment the 4.4 kb HindIII fragment of pDPG325 containing the *bar* expression cassette and 2 kb of the *uidA* expression cassette (structural gene and 3' end) were purified by gel electrophoresis on a 1.2% low melting temperature agarose gel. The 4.4 kb DNA fragment was recovered from the agarose gel by melting gel slices in a 6-10 fold excess of Tris-EDTA buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 70-72C); frozen and thawed (37C); and the agarose pelleted by centrifugation. A Qiagen Q-100 column was used for purification of DNA. For efficient recovery of DNA it was necessary to reduce the flow rate of the column to 40 ml/hr. Isolated DNA fragments can be recovered from agarose gels using a variety of electroelution techniques, enzyme digestion of the agarose, or binding of DNA to glass beads (e.g., Gene Clean). In addition HPLC and/or use of magnetic particles may be used to isolate DNA fragments. This DNA was delivered to AT824 cells using microprojectile bombardment. Twenty four transformants were recovered following selection on bialaphos containing culture medium. No transformants contained the ampicillin resistance gene or origin of DNA replication present in the plasmid vector.  $R_0$  plants have been produced from 11 of these transformed cell lines. Fertility has been demonstrated in plants from ten transformants and  $R_1$  seed has been planted in field tests.

As an alternative to isolation of DNA fragments a plasmid vector can be digested with a restriction enzyme and this DNA delivered to maize cells without

prior purification of the expression cassette fragment. In one experiment pDPG165 was digested with EcoRI and HindIII. This digestion produces an approximately 1900 base pair fragment containing the 35S-*bar*-Tr7 expression cassette and an approximately 2600 base pair DNA fragment containing the ampicillin resistance gene and bacterial origin of DNA replication. This DNA was delivered to AT824 cells using microprojectile bombardment and 2/9 transformants (22%) isolated did not contain the ampicillin resistance gene. In a second experiment pDPG165 digested with restriction enzymes as described above was delivered to AT824 cells via electroporation. Eight of twenty four transformants (33%) recovered lacked the ampicillin resistance gene. Plant regeneration is in progress from transformants lacking the ampicillin resistance gene that were produced in these two experiments.

#### **Example 12: Bombardment of Immature Embryos**

Immature embryos (1.2 - 2.0 mm in length) were excised from surface-sterilized, greenhouse-grown ears of Hi-II 11-12 days post-pollination. The Hi-II genotype was developed from an A188 x B73 cross for high frequency development of type II callus from immature embryos (Armstrong et al., 1991). Approximately 30 embryos per petri dish were plated axis side down on a modified N6 medium containing 1 mg/l 2,4-D, 100 mg/l casein hydrolysate, 6 mM L-proline, 0.5 g/l 2-(N-morpholino)ethanesulfonic acid (MES), 0.75 g/l MgCl<sub>2</sub>, and 2% sucrose solidified with 2 g/l Gelgro, pH 5.8 (#735 medium). Embryos were cultured in the dark for two days at 24° C.

Approximately four hours prior to bombardment, embryos were transferred to the above culture medium with the sucrose concentration increased from 3% to 12%. When embryos were transferred to the high osmoticum medium they were arranged in concentric circles on the plate, starting 2 cm from the center of the dish, positioned such that their coleorhizal end was orientated toward the center of

the dish. Usually two concentric circles were formed with 25-35 embryos per plate.

Preparation of gold particles carrying plasmid DNA was described in example 10. Particles were prepared containing 10 ug pDPG215 (luciferase), pDPG415 (Bt), and pDPG417 (bar) or 30 ug pDPG265 containing the maize R and C1B genes for anthocyanin biosynthesis.

The plates containing embryos were placed on the third shelf from the bottom, 5 cm below the stopping screen. The 1100 psi rupture discs were used. Each plate of embryos was bombarded once. A total of 420 embryos were bombarded on 14 plates with the luciferase, *bar*, and *Bt* genes. Embryos were allowed to recover overnight on high osmotic strength medium prior to initiation of selection. A set of plates was also bombarded with the C1B vector pDPG265. Red spots representing transient expression of anthocyanin pigments are observed 24 hours after DNA introduction.

**Example 13: Electroporation Experiment EP413: Stable Transformation of SC716 and AT824 cells using pDPG165 and pDPG208**

Maize suspension culture cells were enzyme treated and electroporated using conditions described in Krzyzek and Laursen (PCT Publication WO 92/12250). SC716 or AT824 suspension culture cells, three days post subculture, were sieved through 1000  $\mu$ m stainless steel mesh and washed, 1.5 ml packed cells per 10 ml, in incubation buffer (0.2 M mannitol, 0.1% bovine serum albumin, 80 mM calcium chloride, and 20 mM 2-(N-morpholino)-ethane sulfonic acid, pH 5.6). Cells were then treated for 90 minutes in incubation buffer containing 0.5% pectolyase Y-23 (Seishin Pharmaceutical, Tokyo, Japan) at a density of 1.5 ml packed cells per 5 ml of enzyme solution. During the enzyme treatment, cells were incubated in the dark at approximately 25° C on a rotary shaker at 60 rpm. Following pectolyase

treatment, cells were washed once with 10 ml of incubation buffer followed by three washes with electroporation buffer (10 mM HEPES, 0.4 mM mannitol). Cells were resuspended in electroporation buffer at a density of 1.5 ml packed cells in a total volume of 3 ml.

5

Linearized plasmid DNA, 100 ug of EcoRI digested pDPG165 and 100 ug of EcoRI digested pDPG208, was added to 1 ml aliquots of electroporation buffer. The DNA/electroporation buffer was incubated at room temperature for approximately 10 minutes. To these aliquots, 1 ml of suspension culture cells/electroporation buffer (containing approximately 0.5 ml packed cells) were added. Cells and DNA in electroporation buffer were incubated at room temperature for approximately 10 minutes. One half ml aliquots of this mixture were transferred to the electroporation chamber (Puite, 1985) which was placed in a sterile 60 X 15 mm petri dish. Cells were electroporated with a 70, 100, or 140 volt (V) pulse discharged from a 140 microfarad ( $\mu$ f) capacitor.

15

Approximately 10 minutes post-electroporation, cells were diluted with 2.5 ml 409 medium containing 0.3 M mannitol. Cells were then separated from most of the liquid medium by drawing the suspension up in a pipet, and expelling the medium with the tip of the pipet placed against the petri dish to retain the cells. The cells, and a small amount of medium (approximately 0.2 ml) were dispensed onto a filter (Whatman #1, 4.25 cm) overlaying solid 227 medium (Table 1) containing 0.3 M mannitol. After five days, the tissue and the supporting filters were transferred to 227 medium containing 0.2 M mannitol. After seven days, tissue and supporting filters were transferred to 227 medium without mannitol.

20

25

#### Example 14: Electroporation of Immature embryos

Immature embryos (0.4 - 1.8 mm in length) were excised from a surface-sterilized, greenhouse-grown ear of the genotype H99 11 days post-pollination.

5 Embryos were plated axis side down on a modified N6 medium containing 3.3 mg/l dicamba, 100 mg/l casein hydrolysate, 12 mM L-proline, and 3% sucrose solidified with 2 g/l Gelgro®, pH 5.8 (#726 medium), with about 30 embryos per dish. Embryos were cultured in the dark for two days at 24° C.

10 Immediately prior to electroporation, embryos were enzymatically treated with 0.5% Pectolyase Y-23 (Seishin Pharmaceutical Co.) in a buffer containing 0.2 M mannitol, 0.2% bovine serum albumin, 80 mM calcium chloride and 20 mM 2-(N-morpholino)-ethane sulfonic acid (MES) at pH 5.6. Enzymatic digestion was carried out for 5 minutes at room temperature. Approximately 140 embryos were  
15 treated in batch in 2 ml of enzyme and buffer. The embryos were washed two times with 1 ml of 0.2 M mannitol, 0.2% bovine serum albumin, 80 mM calcium chloride and 20 mM MES at pH 5.6 followed by three rinses with electroporation buffer consisting of 10 mM 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 0.4 M mannitol at pH 7.5. For the electroporations, the final rinse of  
20 electroporation buffer was removed and the embryos were incubated with 0.33 mg/ml linearized pDPG165, 0.33 mg/ml supercoiled pDPG215 and 0.33 mg/ml linearized pDPG344 in electroporation buffer. One half ml aliquots of DNA in electroporation buffer and twenty embryos were transferred to the electroporation chamber that was placed in a sterile 60x15 mm Petri dish. An electrical pulse was  
25 passed through the cells from a 500  $\mu$ f capacitor that was charged to 100 volts (400 V/cm field strength, 160 ms pulse decay time; exponential pulse).

Immediately following electroporation, embryos were diluted 1:10 with 726 medium containing 0.3 M mannitol. Embryos were then transferred to Gelgro®  
30 solidified 726 medium containing 0.3 M mannitol. Embryos were incubated in the



dark at 24° C. After five days embryos were transferred to Gelgro solidified 726 medium containing 0.2 M mannitol. Two days later embryos were transferred to selection medium.

## 5      **Example 15: DNA delivery using silicon carbide fibers**

Kaeppler *et al.* 1990 reported transformation of tobacco and BMS suspensions using fibers having a size of 0.6 x 10 to 80  $\mu\text{m}$ . Transformations were accomplished by vortexing the silar fibers together with cells in a DNA solution. DNA passively enters as the cell are punctured. It is contemplated that fibers and/or particles of other types would also be useful. The suspension culture SC82 was tested for transformability using the silicon carbide (silar) transformation method described by Kaeppler *et al.* The initiation of cell line SC82 is described in example 2.

A 2% mixture of silar in absolute ethanol was prepared. Microfuge tubes were prepared (one per sample) by pipetting 80  $\mu\text{l}$  of silar into each tube. The fibers were pelleted and the ethanol removed. Samples were then washed with sterile water, pelleted, and the water removed. Plasmid DNA (25  $\mu\text{l}$  of 1 mg/ml) was added to each tube. Tissue samples were prepared by adding 0.25 ml PCV of cells to a second set of microfuge tubes. Cells were pelleted and the medium removed. A 100  $\mu\text{l}$  aliquot of fresh medium was next added to each tissue sample. The silar/ DNA mixture was resuspended and added to the cells. The transformation was carried out by inverting the microfuge tubes and vortexing for 10 seconds followed by placing the tube upright and vortexing for an additional one minute. Samples were then removed and cultured in small petri dishes with 3 ml of medium. Transient GUS activity was observed two days after DNA delivery.

In a second experiment with SC82 a bead beater or a vortex was utilized to agitate samples. Samples were prepared as described above. ~~Two samples were~~

agent. In the population of surviving cells are those cells wherein generally the resistance-conferring gene has been integrated and expressed at sufficient levels to permit cell survival. Cells may be tested further to confirm stable integration of the exogenous DNA. Using embryogenic suspension cultures, stable transformants are recovered at a frequency of approximately 1 per 1000 transiently expressing foci. A specific embodiment of this procedure is shown in Example 17.

One of the difficulties in cereal transformation, e.g., corn, has been the lack of an effective selective agent for transformed cells, from totipotent cultures (Potrykus, 1989). Stable transformants were recovered from bombarded nonembryogenic Black Mexican Sweet (BMS) maize suspension culture cells, using the *neo* gene and selection with the aminoglycoside, kanamycin (Klein, 1989). This approach, while applicable to the present invention, is not preferred because many monocots are insensitive to high concentrations of aminoglycosides (Dekeyser *et al.*, 1989; Hauptmann *et al.*, 1988). The stage of cell growth, duration of exposure and concentration of the antibiotic, may be critical to the successful use of aminoglycosides as selective agents to identify transformants (Lyznik *et al.*, 1989; Yang *et al.*, 1988; Zhang *et al.*, 1988). For example, D'Halluin *et al.* (1992) demonstrated that using the *neo* gene and selecting with kanamycin transformants could be isolated following electroporation of immature embryos of the genotype H99 or type I callus of the genotype PA91. In addition, use of the aminoglycosides, kanamycin or G418, to select stable transformants from embryogenic maize cultures, in the inventors' experience, often results in the isolation of resistant calli that do not contain the *neo* gene.

One herbicide which has been suggested as a desirable selection agent is the broad spectrum herbicide bialaphos. Bialaphos is a tripeptide antibiotic produced by *Streptomyces hygroscopicus* and is composed of phosphinothricin (PPT), an analogue of L-glutamic acid, and two L-alanine residues. Upon removal of the L-alanine residues by intracellular peptidases, the PPT is released and is a potent

inhibitor of glutamine synthetase (GS), a pivotal enzyme involved in ammonia assimilation and nitrogen metabolism (Ogawa *et al.*, 1973). Synthetic PPT, the active ingredient in the herbicides Basta® or Ignite® is also effective as a selection agent. Inhibition of GS in plants by PPT causes the rapid accumulation of ammonia and death of the plant cells.

The organism producing bialaphos and other species of the genus *Streptomyces* also synthesizes an enzyme phosphinothricin acetyl transferase (PAT) which is encoded by the *bar* gene in *Streptomyces hygroscopicus* and the *pat* gene in *Streptomyces viridochromogenes*. The use of the herbicide resistance gene encoding phosphinothricin acetyl transferase (PAT) is referred to in DE 3642 829 A wherein the gene is isolated from *Streptomyces viridochromogenes*. In the bacterial source organism this enzyme acetylates the free amino group of PPT preventing auto-toxicity (Thompson *et al.*, 1987). The *bar* gene has been cloned (Murakami *et al.*, 1986; Thompson *et al.*, 1987) and expressed in transgenic tobacco, tomato and potato plants (De Block, 1987) and *Brassica* (De Block, 1989). In previous reports, some transgenic plants which expressed the resistance gene were completely resistant to commercial formulations of PPT and bialaphos in greenhouses.

PCT Application No. WO 87/00141 refers to the use of a process for protecting plant cells and plants against the action of glutamine synthetase inhibitors. This application also refers to the use of such of a process to develop herbicide resistance in determined plants. The gene encoding resistance to the herbicide BASTA (Hoechst, phosphinothricin) or Herbiace (Meiji Seika, bialaphos) was said to be introduced by *Agrobacterium* infection into tobacco (*Nicotiana tabacum* cv Petit Havan SR1), potato (*Solanum tuberosum* cv Benolima) and tomato (*Lycopersicum esculentum*) and conferred on plants resistance to application of herbicides.

Another herbicide which is useful for selection of transformed cell lines in the practice of this invention is the broad spectrum herbicide glyphosate. Glyphosate inhibits the action of the enzyme EPSPS which is active in the aromatic amino acid biosynthetic pathway. Inhibition of this enzyme leads to starvation for the amino acids phenylalanine, tyrosine, and tryptophan and secondary metabolites derived thereof. U.S. Patent 4,535,060 describes the isolation of EPSPS mutations which confer glyphosate resistance on the *Salmonella typhimurium* gene for EPSPS, *aroA*. The EPSPS gene was cloned from *Zea mays* and mutations similar to those found in a glyphosate resistant *aroA* gene were introduced in vitro. The mutant gene encodes a protein with amino acid changes at residues 102 and 106. Although these mutations confer resistance to glyphosate on the enzyme EPSPS it is anticipated that other mutations will also be useful.

An exemplary embodiment of vectors capable of delivering DNA to plant host cells is the plasmid, pDPG165 and the vectors pDPG433, pDPG434, pDPG435, and pDPG436.. The plasmid pDPG165 is illustrated in Fig. 1A and 1C. A very important component of this plasmid for purposes of genetic transformation is the *bar* gene which encodes a marker for selection of transformed cells exposed to bialaphos or PPT. Plasmids pDPG434 and pDPG436 contain a maize EPSPS gene with mutations at amino acid residues 102 and 106 driven by the actin promoter and 35S promoter-Adh1 intron I respectively. A very important component of these plasmids for purposes of genetic transformation is the mutated EPSPS gene which encodes a marker for selection of transformed cells.

**Example 16: Selection of *bar* Transformants Using Bialaphos in the Cell Line SC82 Following Particle Bombardment**

The suspension culture (designated SC82) used in the initial experiments (see Example 8) was derived from embryogenic Type-II callus of A188 X B73. Following bombardment (see Example 8), cells on filters were resuspended in nonselective liquid medium, cultured for 1 to 2 weeks and transferred to filters overlaying solid medium containing 1 or 3 mg/l bialaphos. The degree of inhibition of tissue growth during selection was dependent upon the density of the cells on the filter and on the concentration of bialaphos used. At the density plated (0.5 PCV/filter), growth of cells cultured on 1 mg/l bialaphos was only partially inhibited (~30-50% of nonselected growth) and after 3 to 4 weeks much of this tissue was transferred as discrete clumps (~5 mm in diameter) to identical medium. On medium containing 3 mg/l bialaphos, the growth of cells on the original selection filter was severely inhibited (~10% of nonselected growth) and selection was carried out without removing the tissue from the original filter.

Using either selection protocol (1 or 3 mg/l bialaphos), resistant cell colonies emerged on the selection plates of SC82 bombarded with pDPG165 approximately 6 to 7 weeks after bombardment (Fig. 2A). Bialaphos-resistant calli were maintained and expanded on selection medium. Much of this tissue was embryogenic (Fig. 2B). No colony growth occurred on plates to which cells were added from untransformed suspension cultures. These were controls which confirm the prediction that cells without the *bar* gene are not resistant to bialaphos.

Colonies on solid supports are visible groups of cells formed by growth and division of cells plated on such support. Colonies can be seen in Fig. 2A on a petri dish. In this figure, the cells capable of growth are those that are resistant to the presence of the herbicide bialaphos, said resistance resulting from integration and expression of the *bar* gene. Exposure of cells was to 1 mg/l bialaphos. Figure 2B

is a magnification showing the morphology of one bialaphos-resistant culture maintained on selection media indicating that growth is embryogenic.

As a confirmation that the cells forming the colonies shown in Fig. 2 had indeed incorporated the *bar* gene and were expressing it, bialaphos-resistant callus lines were analyzed for activity of the *bar* gene product, phosphinothricin acetyl transferase (PAT), by thin-layer chromatography. Protein extracts from eleven callus lines (E1-11) isolated from SC82 bombardment experiments contained PAT activity as shown in Figure 3 and activity levels varied approximately 10-fold among the isolates.

Still further and more direct confirmation of the presence of the *bar* gene was obtained by analysis of the genomic DNA of potential transformants by DNA gel blots (Figure 4). The sources of DNA which were electrophoresed through the gel were the bialaphos-resistant callus lines designated E1-E11 and a non-selected control, EO. (Fig. 1 indicates the cleavage sites of those enzymes within the *bar* gene plasmid). After the DNA was electrophoresed through the gel and transferred to nylon membranes, the resulting blot was hybridized with a  $^{32}\text{P}$ -labeled *bar* gene sequence from the plasmid pDPG165. The radioactivity used per blot was approximately  $25 \times 10^6$  Cerenkov cpm. The lane in Figure 4 designated "1" and "5" copies contain 1.9 and 9.5 pg respectively of the 1.9 kb *bar* expression unit released from the plasmid pDPG165 by application of the EcoRI and HindIII enzymes; these amounts represent about 1 and 5 copies per diploid genome.

Genomic DNA from all eleven bialaphos-resistant isolates contained *bar*-hybridizing sequences as shown in Figure 4. The hybridization in all isolates to a fragment migrating slightly larger than 2 kb may be due to contaminating pUC19 sequences contained in this *bar* probe preparation; no such hybridization occurred in subsequent experiments using the same genomic DNA and a different preparation of the *bar* probe. Hybridization to a 1.9 kb fragment in eight of the

eleven isolates indicated that these isolates contained intact copies of the 1.9 kb *bar* expression unit. The estimated copy numbers of the intact unit ranged from one or two (E1, E7, E8, E10, E11) to approximately 20 (E3, E4, E6). Hybridization with the *bar* probe in isolates E2 and E5 occurred only to a single, higher molecular weight fragment (~3 kb).

To establish that the PAT coding sequence was intact in isolates E2 and E5, genomic DNA was digested with *Sma*I, which releases a 559 bp fragment containing the PAT structural gene (Figure 1A), and subjected to DNA gel blot analysis using <sup>32</sup>P-labeled *bar*. This analysis confirmed the presence of a single intact copy of *bar*. Expression of PAT in these isolates may not be dependent on the 35S promoter or the Tr7 3' end. The hybridization patterns of some of the isolates were identical (E2 and E5; E7 and E8; E3, E4, and E6); therefore, it is probable that some isolates did not arise from independent transformation events but represent transformants that were separated during selection.

Seven hybridization patterns were unique, likely representing seven independent single-cell transformation events. The patterns and intensities of hybridization for the seven transformants were unchanged during four months in culture, providing evidence for the stability of the integrated sequences. The seven independent transformants were derived from two separate bombardment experiments. Four independent transformants representing isolates E2/E5, E3/E4/E6, E1 and E7/E8, were recovered from a total of four original filters from bombardment experiment #1 and the three additional independent transformants, E9, E10, and E11, were selected from tissue originating from six bombarded filters in experiment #2. These data are summarized in Table 5.

Table 5

Summary of Maize Transformation Experiments

5

Exp. 10 #	Culture Bombarded	# of Filters Bombarded	# of Independent <i>bar</i> Transformants Recovered	# with Intact <i>bar</i> Expression Units	# with GUS Coding Sequence	# with GUS Activity	Cointegration Frequency (%)	Coexpression Frequency (%)
1	SC82	4	4	3	n.a			
2	SC82	6	3	2	n.a.			
3	SC94	10	8	6	n.a.			
4	SC716*	8	13	8	11	3	85	23
15 5	SC716*	8	7	4	6	1	86	14
6	SC82*	4	19	17	13	3	68	16
TOTALS		40	54	40	30	7	77(30/39)	18(7/39)

20 culture reinitiated from cryopreserved cells

n.a. not applicable; only pDPG165 DNA used or co-transformation analysis not done





**Example 18:        Assays for Integration  
and Expression of GUS**

SC716 transformants discussed in Example 17, were further analyzed for  
5 integration and expression of the gene encoding GUS. As determined by  
histochemical assay, four of the SC716 transformants (R5, R7, R16, and R21) had  
detectable GUS activity 3 months post-bombardment. Expression patterns  
observed in the four coexpressing callus lines varied. The number of cells with  
GUS activity within any given transformant sampled ranged from ~5% to ~90%  
10 and, in addition, the level of GUS activity within those cells varied. The  
cointegration frequency was determined by washing the genomic blot hybridized  
with *bar* (Figure 5A) and probing with <sup>32</sup>P-labeled GUS sequence as shown in  
Figure 5B. EcoRI and HindIII, which excise the *bar* expression unit from pDPG165,  
also release from pDPG208 a 2.1 kb fragment containing the GUS coding sequence  
15 and the *nos* 3' end (Figure 1B).

Seventeen of the independent *bar* transformants contained sequences that  
hybridized to the GUS probe; three, R2, R14 and R19 did not. Transformants in  
which GUS activity was detected (R5, R7, R16 and R21) had intact copies of the  
20 2.1 kb EcoRI/HindIII fragment containing the GUS structural gene (Figure 5B).  
Transformants that contained large numbers of fragments that hybridized to *bar*  
(R1, R5, R21) also contained comparable number of fragments that hybridized to  
the gene encoding GUS (Figures 5A and B). This observation is consistent with  
those reported using independent plasmids in PEG-mediated transformation of  
25 A188 X BMS protoplasts (Lyznik *et al.*, 1989) and in studies conducted by the  
inventors involving bombardment-mediated transformation of BMS suspension  
cells.

**Example 19: Transformation of Cell Line AT824 Using Bialaphos Selection**  
**Following Particle Bombardment--Selection in Liquid Medium**

The suspension culture (designated AT824) used in this experiment was  
5 derived from an elite B73-derived inbred (described in example 3). The culture was  
maintained in medium 409. Four filters were bombarded as described in example  
10.

Following one week culture in liquid medium 409 without selection pressure,  
10 tissue was transferred to liquid medium 409 containing 1 mg/L bialaphos. Cells  
were transferred twice per week into fresh medium containing 1 mg/L bialaphos for  
two weeks. Tissue was then planted 3 weeks following bombardment at a  
concentration of 0.1 ml packed cell volume per petri dish containing medium 425  
(with 3 mg/L bialaphos). Transformants were identified as discreet colonies 6  
15 weeks following bombardment. It is the experience of the inventors that all cell  
lines that grow on 3 mg/L bialaphos contain the *bar* gene. Fifty transformed cell  
lines were recovered from this experiment. Twenty four of these cell lines  
contained the *Bt* gene.

**Example 20: Transformation of Cell Line AT824 Using Bialaphos Selection**  
**Following Particle Bombardment--Solid Medium Selection**

Cells in experiment S10 were bombarded as described in example 10 except  
the gold particle-DNA preparation was made using 25 ul pDPG319 DNA (*bar* gene  
25 and *aroA* expression cassette containing the  $\alpha$ -tubulin promoter). Following particle  
bombardment cells remained on solid 279 medium in the absence of selection for  
one week. At this time cells were removed from solid medium, resuspended in  
liquid 279 medium, replated on Whatman filters at 0.5 ml PCV per filter, and  
transferred to 279 medium containing 1 mg/L bialaphos. Following one week,  
30 filters were transferred to 279 medium containing 3 mg/L bialaphos. One week  
later, cells were resuspended in liquid 279 medium and plated at 0.1 ml PCV on

279 medium containing 3 mg/L bialaphos. Nine transformants were identified 7 weeks following bombardment.

#### **Example 21: Transformation of Cell Line ABT4 Using Bialaphos Following Particle Bombardment**

Initiation of cell line ABT4 is described in example 4. ABT4 was maintained as a callus culture. At the time of subculture, tissue was scraped off the solid culture medium and resuspended in 20 mls of 708 medium containing 0.2M mannitol. Tissue was dispersed with a large bore 10 ml pipette by picking up and dispensing several times until one could pickup 0.5 ml packed cell volume (PCV) for subculture to fresh solid 708 medium. Prior to bombardment three week old 708 maintenance cultures of ABT4 were transferred from solid medium to 20 mls 708 + 0.2M mannitol and 0.5 ml PCV was plated on glass fiber filters over 708 + 0.2M mannitol medium. Cultures were allowed to plasmolyze for 2-4 hours prior to bombardment. At the time of bombardment tissue on a glass fiber filter was placed on top of 3 filter papers moistened with 2.5 mls of 708 + 0.2M mannitol. Six ul of DNA/gold particles (described in example 10) was placed on flyers prior to bombardment with the Dupont Biolistics PDS1000He particle delivery device. Particles were accelerated by a 1100 psi blast of helium gas. Following bombardment tissue was returned to 708 + 0.2M mannitol and allowed to recover for 2-5 days. Selection began at this point by moving the tissue/filter to 708 + 1 mg/L bialaphos for 12 days. At this time tissue was transferred to 30-40 ml 708 + 0.5 mg/L bialaphos, dispersed, and thin plated at 0.05 to 0.10 PCV on 708 + 0.5 mg/L bialaphos solid medium. Transformants were identified 5-12 weeks following thin plating. Following identification transformants were maintained on 708 + 3 mg/L bialaphos.

## **Example 22: Transformation of Immature Embryos of the Genotype Hi-II Using Bialaphos as a Selective Agent Following Particle Bombardment**

Immature embryos of the genotype Hi-II were bombarded as described in example 12. Embryos were allowed to recover on high osmoticum medium (735, 12% sucrose) overnight (16 - 24 hours) and were then transferred to selection medium containing 1 mg/l bialaphos (#739, 735 plus 1 mg/l bialaphos or #750, 735 plus 0.2M mannitol and 1 mg/l bialaphos). Embryos were maintained in the dark at 24° C. After three to four week on the initial selection plates about 90% of the embryos had formed Type II callus and were transferred to selective medium containing 3 mg/l bialaphos (#758). Responding tissue was subcultured about every two weeks onto fresh selection medium (#758). Nineteen transformants were identified six to eight weeks after bombardment. Fifteen of nineteen transformants contained the *B. thuringiensis* (*Bt*) crystal toxin gene. Plants have been regenerated from one transformant containing the *Bt* gene and transferred to soil in the greenhouse. Regeneration of plants from remaining lines containing the *Bt* gene is in progress.

## **Example 23: Transformation of AT824 and SC716 Using Bialaphos Selection Following Electroporation**

Cells of AT824 and SC716 were electroporated and allowed to recover from electroporation as described in example 13. Five days later, tissue growing on filters was removed from the filter and transferred as clumps (approximately 0.5 cm in diameter) to the surface of solid selection medium. The selection medium consisted of 227 medium supplemented with 1 mg/L bialaphos. Three weeks later, slowly growing tissue was transferred, as 0.5 cm clumps, to 227 medium containing 3 mg/L bialaphos. Three to four weeks later, callus sectors that continued to grow were transferred to fresh 227 medium containing 3 mg/L bialaphos. Callus lines that continued to grow after this subculture were considered to be transgenic and perpetuated further, by transfer to fresh selection

medium every two weeks. One SC716 and seven AT824 callus lines were selected in this example. The SC716 callus line was recovered from an electroporation at 140  $\mu$ f, 100 V. Three AT824 callus lines were recovered from 140  $\mu$ f, 70 V electroporations and four AT824 callus lines were recovered from electroporation at 140  $\mu$ f, 140 V.

Three bialaphos resistant callus lines selected in this example, one derived from SC716 and two derived from AT824, were randomly chosen and assayed for phosphinothricin acetyltransferase (PAT) activity. PAT is the *bar* gene product, and PAT activity is determined by the ability of total protein extracts from potentially transformed cells to acetylate phosphinothricin (PPT), using  $^{14}$ C-acetyl coenzyme A as the acetyl donor. This transfer is detected, using thin layer chromatography and autoradiography, by a shift in the mobility of  $^{14}$ C labelled compound from that expected for  $^{14}$ C-acetyl coenzyme A to that expected for  $^{14}$ C-N-acetyl PPT. The assay used for detection of PAT activity has been described in detail (Adams et al., published PCT application no. WO91/02071; Spencer et al.1990). All three callus lines tested contained PAT activity.

In this example, suspension culture cells were electroporated with a second plasmid, pDPG208, encoding  $\beta$ -glucuronidase (GUS). Detection of GUS activity can be performed histochemically using 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) as the substrate for the GUS enzyme, yielding a blue precipitate inside of cells containing GUS activity. This assay has been described in detail (Jefferson1987). One of the seven AT824 callus lines selected in this example, EP413-13, contained cells that turned blue in the histochemical assay. The callus line derived from SC716 in this example did not contain detectable GUS activity.

Southern blot analysis was performed on three bialaphos resistant callus lines to determine the presence and integration of the *bar* gene in genomic callus DNA. Southern blot analysis was performed as follows. Genomic DNA was isolated using a procedure modified from Shure et al. (1983). Approximately one

gram of callus tissue from each line was lypholyzed overnight in 15 ml polypropylene tubes. Freeze-dried tissue was ground to a powder in the tube using a glass rod. Powdered tissue was mixed thoroughly with 3 ml extraction buffer (7.0 M urea, 0.35 M NaCl, 0.05 M Tris-HCl pH 8.0, 0.01 M EDTA, 1% sarcosine).  
5 Tissue/buffer homogenate was extracted with 3 ml phenol/chloroform. The aqueous phase was separated by centrifugation, and precipitated twice using 1/10 volume of 4.4 M ammonium acetate pH 5.2, and an equal volume of isopropanol. The precipitate was washed with 75% ethanol and resuspended in 100-500  $\mu$ l TE (0.01 M Tris-HCl, 0.001 M EDTA, pH 8.0). Genomic DNA was digested with a 3-  
10 fold excess of restriction enzymes, electrophoresed through 0.8% agarose (FMC), and transferred (Southern, 1975) to Nytran (Schleicher and Schuell) using 10X SCP (20X SCP: 2 M NaCl, 0.6 M disodium phosphate, 0.02 M disodium EDTA). Filters were prehybridized in 6X SCP, 10% dextran sulfate, 2% sarcosine, and 500  $\mu$ g/ml heparin (Chomet et al., 1987) for approximately 10 minutes. Filters were hybridized  
15 overnight at 65 °C in 6X SCP containing 100  $\mu$ g/ml denatured salmon sperm DNA and <sup>32</sup>P-labelled probe. Probe was generated by random priming (Feinberg and Vogelstein, 1983); Boehringer-Mannheim). Hybridized filters were washed in 2X SCP, 1% SDS at 65° for 30 minutes and visualized by autoradiography using Kodak XAR5 film.

20 In this example, genomic DNA isolated from bialaphos resistant callus lines was digested with HindIII and EcoRI, which release a 1.9 kb *bar* fragment from pDPG165 (Figure 1A). Genomic DNA was probed with <sup>32</sup>P labelled 0.6 kb SmaI *bar* fragment from pDPG165 (Figure 1A). All three EP413 callus lines analyzed  
25 contained DNA that hybridized to the *bar* probe. Copy number in the transformed callus ranged from one to two copies (EP413-3) to greater than 20 copies of *bar* (EP413-1). Furthermore, the restriction digest used, yielded *bar*-hybridizing fragments in callus DNA samples that were larger than the *bar* fragment released from pDPG165 in the same restriction digest. This result is indicative of stable  
30 integration of introduced DNA into the maize genome.

Thirty-nine plants were regenerated from seven of the eight bialaphos resistant callus lines selected in this example. Plants were regenerated from six AT824 callus lines and the single SC716 callus line. The plant regenerated from the SC716 callus line (EP413-4) did not survive to maturity. For plant  
5 regeneration, callus growing on 227 medium containing 3 mg/L bialaphos, was transferred to 189 medium (Table 1). Somatic embryos matured on 189 medium after one, two, or three two week subculture periods in the dark at 25 °C. As somatic embryos developed on 189 medium, clumps of tissue containing these embryos were transferred to growth regulator free 101 medium (Table 1) and  
10 placed in the light ( $25\text{-}250\ \mu\text{E M}^{-2}\ \text{s}^{-1}$ ). Plantlets developed on this medium after one, two, or three subculture periods. Plantlets were subsequently transferred to 501 medium (Table 1) in Plant Con<sup>R</sup> containers for rooting and further growth. Regenerates ( $R_0$  plants) were subsequently transferred to a soilless mix in 0.5 liter pots and acclimated to ambient humidity in a growth chamber ( $200\text{-}450\ \mu\text{E M}^{-2}\ \text{s}^{-1}$ ;  
15 14 h photoperiod). The soilless mix has been described in detail (Adams et al., published PCT application no. WO91/02071). Plants were then transferred to a soilless mix in 16 liter pots and grown to maturity in a greenhouse.

Plants regenerated from five different EP413 callus lines were assayed for  
20 PAT activity as described for callus earlier in this example. All five plants contained PAT activity. Three plants regenerated from the single EP413 callus line that exhibited GUS activity (EP413-13) were analyzed for GUS activity. All three EP413-13  $R_0$  plants were positive for GUS activity. Files of blue cells were observed in leaf tissue of EP413-13 plants upon incubation with X-Gluc.

EP413  $R_0$  plants were also analyzed for the presence and integration of *bar*  
by Southern blot. DNA was isolated from leaf tissue as described for callus except that fresh, rather than lypholyzed tissue was used. Prior to the addition of  
25 extraction buffer, fresh leaf tissue was frozen in liquid nitrogen and ground to a  
30 fine powder in a 15 ml polypropylene tube using liquid nitrogen and a glass rod. DNA was isolated from four EP413  $R_0$  plants, each representing a different callus



line. DNA was analyzed, digested with HindIII and BglII, or undigested, for hybridization to *bar*. R<sub>0</sub> DNA was probed with <sup>32</sup>P labelled 0.6 kb SmaI *bar* fragment from pDPG165 (Figure 1A). HindIII/BglII digestion of pDPG165 releases a fragment containing 35S-*bar* of approximately 1.3 kb (Figure 1A). Genomic DNA  
5 from all four plants contained at least one copy of the 1.3 kb HindIII/BglII 35S-*bar* fragment. In addition, undigested genomic DNA from all four plants exhibited hybridization to *bar* only in high molecular weight DNA (> 20 kb), indicating integration of pDPG165 into maize chromosomal DNA.

10 Progeny were recovered from outcrosses made between electroporation-derived, transgenic R<sub>0</sub> plants and non-transformed inbred plants. Four EP413-3 R<sub>0</sub> plants were the first of the plants to reach maturity and flower. One of the plants was outcrossed as the male to a CD inbred plant. This cross resulted in 22  
15 kernels. Sixteen of these kernels were planted in soilless mix and all germinated. Approximately two weeks post-germination, the progeny (R<sub>1</sub>) plants were analyzed for PAT activity. Three of sixteen plants contained PAT activity. Four transgenic EP413-3 R<sub>0</sub> plants were also outcrossed as the female, using pollen collected from nontransformed inbred plants. Kernels developed on ears of  
20 all four EP413-3 R<sub>0</sub> plants. Thirty-seven kernels were recovered from an ear on an EP413-3 R<sub>0</sub> plant treated with pollen collected from a seed-derived, non-transformed FBLL inbred plant. Sixteen of these kernels were planted in soil and twelve germinated. Eight of these plants were analyzed for PAT activity; three of eight were positive  
25 for PAT activity.

These eight plants were also analyzed by Southern blot hybridization for the presence of *bar*. Genomic DNA isolated from these eight EP413-3 R<sub>1</sub> plants was digested with restriction enzymes HindIII and BglII, which release a 1.3 kb fragment  
30 containing *bar* from pDPG165 (Figure 1A). In addition to DNA from the eight EP413-3 R<sub>1</sub> plants, DNA isolated from EP413-3 callus and DNA from the EP413-3

R<sub>0</sub> plant yielding these eight R<sub>1</sub> plants was included in the analysis. Genomic DNA was probed with <sup>32</sup>P labelled 0.6 kb SmaI *bar* fragment from pDPG165 (Figure 1A). Hybridization to *bar* was detected in the DNA isolated from callus, R<sub>0</sub> and the three R<sub>1</sub> plants that contained PAT activity. Each of the *bar*-positive plants contained the expected 1.3 kb HindIII/BglII fragment from pDPG165 (Figure 1A) as well as an additional, larger *bar*-hybridizing fragment of approximately 2.0 kb. This result, as well as the PAT activity found to be present in EP413-3 R<sub>1</sub> plants, conclusively demonstrates the sexual transmission to progeny of a functional gene introduced into maize cells by electroporation.

#### **Example 24: Transformation of H99 Immature Embryos Using Bialaphos as the Selective Agent Following Electroporation**

Immature embryos of H99 were electroporated as described in example 14. Five days after electroporation embryos were transferred to Gelgro solidified 726 medium containing 0.2 M mannitol. Two days later embryos were transferred to selection medium, 726 medium containing 1 mg/l bialaphos, 16 embryos per dish.

Embryos were cultured in the dark at 24<sup>0</sup> C for about seven weeks. Seventy-eight of the approximately one hundred and twenty embryos plated on selection medium produced Type I callus. All responding callus was transferred to modified MS-based medium containing 1 mg/l NAA, 1 mg/l BAP and 3% sucrose solidified with 8 g/l Bactoagar (20 medium) for maturation. After about two weeks the tissue was transferred to 20 medium containing 1 mg/l bialaphos. Two weeks later the tissue was transferred to a modified MS-based medium containing 0.5 mg/l NAA, 0.5 mg/l BAP and 2% sucrose solidified with 8 g/l Bactoagar (7 medium) with 1 mg/l bialaphos. For rooting the tissue was transferred to a modified MS-based medium containing 0.25 mg/l NAA, 0.25 mg/l BAP and 2% sucrose solidified with 8 g/l Bactoagar and finally 1/2 strength MS0.

Tissue from twenty-three of initial seventy-eight responding embryos survived the regeneration and selection and produced plants. A total of seventy-five plants were transferred to soil. Four of the plants died and the remaining seventy-one plants were transferred to the greenhouse.

5

Plants in the greenhouse were tested for the presence of the *bar* gene by PCR analysis of DNA extracted from leaf tissue or by painting the leaves with 2% Basta. One PCR positive plant from embryo # 31 was identified. All of the other plants were either PCR negative or showed severe necrosis in the Basta painting assay. DNA from leaf tissue of plant 3101 was further analyzed by Southern blot hybridization and gave a positive signal when probed with the *Sma* I fragment of pDPG165. This plant was selfed on June 7 and June 8 and backcrossed on June 9, 1993. Nine seed were harvested from this plant. All seed germinated and seven of nine R<sub>1</sub> plants contain the *bar* gene as determined by PCR analysis.

15

#### **Example 25: Transformation of AB12 Using Hygromycin as a Selective Agent Following Particle Bombardment**

AB12 callus was bombarded as described in example 9. Callus was transferred (ten 25 mg clumps per plate) onto 734 medium (see Table 1) containing 15 mg/l hygromycin B (Calbiochem) immediately after bombardment. After 14 days all tissue was transferred to round 2 selection plates that contained 60 mg/l hygromycin. After 21 days on the round 2 selection plates, most of the material was transferred to F medium containing 60 mg/l hygromycin (round 3 selection plates). Both round 2 and round 3 plates were then observed periodically for the appearance of viable sectors of callus. Putative transformed callus line PH1 was observed on a round 2 plate, 70 days after bombardment. Putative transformants PH2 and PH3 were observed on round 3 plates, 58 and 79 days after bombardment, respectively. Lines were then maintained on F medium containing 60 mg/l hygromycin. Plant regeneration and analysis of transformants are described in Walters et al. (1992). Fertile transgenic plants were regenerated and

30

transmission of the chimeric gene for hygromycin resistance was demonstrated through two complete generations.

**Example 26: Transformation of AT824 Using Glyphosate as A Selective Agent  
Following Particle Bombardment**

A mutant maize EPSPS gene was introduced into AT824 suspension culture cells via particle bombardment as described in example 10. In this example, the mutant maize EPSPS gene was carried by plasmid pDPG436. Plasmid pDPG436 contains a maize EPSPS gene with two amino acid changes, Thr to Ile at position 102 and Pro to Ser at position 106. In this plasmid, the mutant maize EPSPS expression cassette contains a 35S promoter/adh1 intron I combination and the nos 3' end. Following bombardment with gold particles coated with pDPG436, AT824 cells were cultured on 279 medium (Table 1) for four days. Subsequently, the cells were returned to liquid 401 medium (Table 1), at a density of 2 ml packed cell volume (PCV) per 20 ml, and cultured for four days. The cells were then transferred, at a density of 2 ml PCV/20 ml, to fresh 401 medium containing 1 mM glyphosate and cultured for four days. The subculture into 401 plus 1mM glyphosate was repeated and after four days the cells were plated at a density of 0.1 ml PCV per 100 X 15 mm petri dish containing 279 plus 1mM glyphosate. Six to eight weeks after bombardment, glyphosate resistant colonies were removed from the selection plates and subcultured onto fresh 279 plus 1 mM glyphosate. Seven glyphosate resistant callus lines were recovered in this example, at a frequency of zero to seven callus lines per bombardment. Two randomly chosen callus lines were analyzed for the presence of the introduced DNA by Southern blot hybridization (see example 23). Genomic DNA isolated from the callus lines was analyzed undigested or digested with *NotI*, which releases the mutant EPSPS expression cassette from pDPG436. The callus DNA was probed with <sup>32</sup>P-labelled *nos* fragment. The *nos* fragment was isolated as an approximately 250 bp *NotI/XbaI* fragment from pDPG425. The *nos* fragment was chosen as the probe to avoid background hybridization possible using an EPSPS probe due to the presence

of an endogenous maize EPSPS gene. Genomic DNA from both callus lines was positive for hybridization to *nos* in both the undigested and *NotI*-digested samples. Both callus DNA samples contained *nos*-hybridizing bands identical in size to the 35S/*adh1* intron I - EPSPS - *nos* fragments released from pDPG436 upon digestion with *NotI*, as well as additional *nos*-hybridizing bands.

In a second experiment, AT824 cells were bombarded with a mutant EPSPS gene under control of the rice actin promoter and intron (Cao et al., Plant Cell Rep (1992) 11:586-591). The plasmid used, pDPG434, contains the rice actin 5' region, the mutant EPSPS gene described in the previous example, and the *nos* 3' end. Bombarded AT824 cells were cultured and selected as described in the previous example. Thirteen glyphosate resistant callus lines were isolated in this example. Four to seven glyphosate resistant callus lines were recovered per bombardment in this example. As in the previous example, two randomly chosen callus lines were analyzed for the introduced DNA by Southern blot hybridization. Using the same analysis as in the previous example, both callus lines were found to contain DNA sequence that hybridized to the *nos* probe, confirming introduction of, and selection for expression of, the introduced mutant EPSPS gene.

#### **H. Identification of Transformed Cells Using Screenable Markers**

In addition to selectable markers such as the *bar* and *aroA* genes, various screenable marker genes have been employed by the present inventors in maize transformation. It is contemplated that screenable markers may be used to ultimately achieve three objectives: (1) the detection of expressing colonies in a population, which may not necessarily employ a visible marker; (2) the visualization, by microscope or unaided eye, of expressing cells within a population or tissue; and (3) the ability to assess tissue- and/or cell-specific expression in gene expression studies. A screenable marker which meets either objective would be useful and one that meets both criteria would be particularly advantageous. Of the potential candidates being considered as screenable markers, luciferase (Example

27) and aequorin satisfy only the first requirement, while modified extension (Example 28) and tyrosinase could potentially meet both goals.

### **Tyrosinase**

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The tyrosinase gene is considered to be a potential screenable marker. Normally, melanin production requires the expression of two genes which encode for tyrosinase and a  $\text{Cu}^{++}$  transfer protein. Recently, an *E. coli* transformant was isolated by Claudio Denoyo (Pfizer) in which the Cu-transfer protein does not appear to be required. There is still a requirement for copper as a coenzyme for tyrosinase, but this is satisfied by 1 mM  $\text{Cu}^{++}$  and the tyrosinase acts as a copper scavenger. The gene itself is small with a high GC content which should be expressed in maize.

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### **Aequorin**

This gene was cloned by Dr. M. Cormier (University of Georgia) and encodes a protein called apoaequorin that is normally produced in jellyfish. When this protein complexes with a class of lipophilic fluorophores referred to as coelenterazines, the activated complex becomes sensitive to  $\text{Ca}^{++}$ . When the complex comes into contact with  $\text{Ca}^{++}$ , the coelenterazine is reduced to an amide and a photon of light is emitted. Thus, this gene encoded the proteinaceous portion of a calcium-sensitive bioluminescent complex.

20

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This gene has been placed behind the 35S promoter and used to generate aequorin expressing tobacco plants which are being employed to study calcium levels in plant tissue. However, there are certain technical difficulties with developing this system into a screenable marker. Firstly, coelenterazines are difficult to obtain. Secondly, the intensity of the bioluminescence emitted by this complex is probably an order of magnitude lower than luciferase and the detection systems needed to visualize this reaction are very sophisticated and expensive.

30

However light emission from aequorin expressing cells have never been measured while the cells were being flooded with both coelenterazine and  $\text{Ca}^{++}$ . This is an important point. The apoaequorin protein is not the rate limiting factor in this reaction, it is the regeneration of reduced coelenterazine from the coelenteramide. Thus there is a requirement for a strong reducing agent in the assay. Using 1% DMSO, coelenterazine and calcium could drive the light emission up to detectable levels. Conversely, when these substrates are not present, i.e. in the plant, this normally energy-requiring reaction would not be occurring.

10 **Example 27: Luciferase as a Screenable Marker**

The *lux* gene, encoding firefly luciferase, was initially tested as a potential screenable marker using C16 protoplast electroporation to evaluate transient expression, and cotransformation of BMS. Using X-ray film to detect bioluminescence, transient expression in C16 protoplasts was detected, but expression in BMS was not high enough for detection on X-ray film. Published results on luciferase expression in tobacco (Ow *et al.*, 1986), indicate that as few as 350 expressing cells could be viably detected.

Two technical developments prompted the inventors to re-examine the feasibility of using this marker. The first is a Polaroid ASA 20,000 ELISA-type film detection system that is easy to use, with sensitivity comparable to, or slightly more sensitive than, X-ray film. The second is a new Luciferase Assay System (Promega), which through the oxidation of luciferyl-CoA, as opposed to luciferin, is claimed to provide a light reaction with greater total intensity and with a greatly extended half-life.

While the scintillation counter and multiwell luminometer afford one means of testing the utility of luciferase screening, i.e. populational screening for bioluminescence, it would be ideal to be able to visualize transformants on the tissue culture plate. This would be even more valuable if the method was not

limited to specific cultures and/or tissue types, and if it could be extended to the whole plant (i.e. for gene expression studies). Computer-enhanced video microscopy has been recognized as a potentially valuable tool for these applications. Recently, the Photon Counting Camera (Hamamatsu) has provided a new level of sensitivity for the video-imaging of bioluminescence.

Bombarded E1 suspension cells were assayed using both the scintillation counter and the Polaroid detection system. Extracting 1/4 of the cells on a filter 48 hours after bombardment, luciferase activity was at the lower limit of detection using the scintillation counter. Using much smaller aliquots of cells, due to the microtiter wells in the assay system, no discernible activity was observed using the Polaroid detection system.

Using dilutions of purified luciferin in both the scintillation counter and the Polaroid system, and comparing these results to scintillation counts of transient *lux* expression after bombardment of E1 cells, it was estimated that there is probably only a 10-fold discrepancy between the transient expression levels and the ability to detect a signal using this film. Further studies were thus conducted to bridge this detection gap. The luciferase system was optimized both with respect to the assay mixture and also with the creation of further luciferase expression vectors.

### **Luciferase Assay Mixture**

The first step in this process was to re-evaluate the composition of the assay mixture, both in terms of relative luminescence and subsequent viability of the tissue. The addition of coenzyme A to the reaction mix has been reported to improve the bioluminescence kinetics of the luciferase assay. This has been one of the features incorporated into the Luciferase Assay System sold by Promega. In comparing the Promega mixture to the luciferase assay mixture usually employed, no significant difference was observed in assaying purified luciferase enzyme. However, when cell extract from a transformed E1 callus line was used, the



Promega mixture produced approximately an 18-fold increase in signal (measured over a two minute period using the scintillation counter).

Multiple experiments were performed to assess the influence of the components of these assay mixtures; varying both the species and concentration of reducing agent, salts, coenzymes, protective proteins, and the substrate itself, luciferin. The type and amount of reducing agent was a point of concern for tissue viability, so two alternatives were compared for signal strength and tissue viability. Both glutathione (i.e. 10-50 mM) and DTT (5-33 mM) were found to produce strong bioluminescence signals in intact cell clusters, although the results were more variable with glutathione. 5 mM DDT provided the best compromise for enhanced signal strength and growth of callus after exposure to the mixture for 20 minutes. The most effective combination found to date is a hybrid, taking components from both the Promega recipe and the previously used standard mixture. This combination resulted in a 2-4 fold increase in signal over the Promega mixture. The recipe for this improved mix is:

25 mM Tris, PO<sub>4</sub> (pH 7.8), 1% BSA (Fraction 5), 5 mM DTT, 1 mM EDTA, 0.3 mM ATP, 8 mM MgCl<sub>2</sub>, 0.47 mM firefly luciferin, 0.3 mM coenzyme A.

#### **Detection of Luciferase Expression**

Once the assay mixture had been optimized, the scintillation counter and luminometer were evaluated as to their utility for screening of transformants. Using cell clusters ranging between 100 to 200 um in diameter, both instruments were capable of detecting luciferase activity in transformants with high expression levels. The luminometer was more sensitive, being able to detect transformants with lower expression levels and/or smaller groups of cells consistently. Plastic covers for the multiwell dishes were obtained that have a minimal effect on reducing the bioluminescent signal, and allow this assay to be performed under sterile conditions.

Reconstruction studies were initiated, placing 10-15 small transformed tissue pieces (all below 150  $\mu\text{m}$  diameter) into 0.75 ml of non-transformed suspension cells. Initial screening was encouraging as luciferase activity could be detected even within this large non-transformed population. This also illustrated the advantages and disadvantages of the two detection devices; the scintillation counter is convenient for screening large aliquots of cells, while the luminometer is more sensitive but more labor intensive.

To take advantage of the relative merits of both devices, in the second reconstruction experiment the scintillation counter and then the luminometer were used for sequential screening. Again, 10-15 transformed cell clusters were mixed into a non-transformed population (1.25 ml of suspension) and placed on the shaker for 2 hours. The suspension was then pipetted into seven scintillation vials and assayed for luciferase activity. Positive signals were recorded for 5/7 samples, and these five were pipetted onto fresh 227 solid medium and allowed to grow for one week. At this time, two of the samples were aliquoted into multiwell dishes and assayed for activity using the luminometer. Each of these wells contained approximately 25  $\mu\text{l}$  of tissue. Six and 7 positive wells were recorded for these two samples, and the tissue again was transferred back onto fresh 227 medium.

These results exemplify the power of the luminometer, because the sample size in each well is small the enrichment is much greater (this single screen eliminated approximately 95% of the population). The drawback to this type of screening is the amount of tissue manipulation and risk of contamination. Despite some contamination it is extremely encouraging that the enrichment technique was successful and that the tissue remained healthy (based on visual assessment and subsequent growth).

## Improved Luciferase Expression Vectors

Recent improvements in the luciferase assay mixture increased the sensitivity enough so that detection of stable transformed sectors appeared feasible. To further improve the chances of successfully screening for transformants, expression should also be optimized. Towards this goal, two new luciferase vectors were constructed to boost expression levels in maize cells. Both vectors utilize intron VI from *Adh1* (derived from vector pDPG273) fused to firefly luciferase (obtained from vector pDPG215). These elements were inserted into either the pDPG282 (4 OCS inverted-35S) or the pDPG283 (4 OCS-35S) backbone (*bar* was excised as a *BamHI/NheI* fragment and the intron plus luciferase gene inserted). The 4 OCS-35S promoter has been shown with the *uidA* gene to give very high levels of transient expression. When this promoter is fused to luciferase it was anticipated that it would result in high levels of expression.

Transient expression levels from each of these vectors was determined in bombarded E1 suspension culture cells. The two new vectors were also compared to pDPG215 (35S-intron I-luciferase-Tr7 3') as the standard. At least for transient expression, the 4 OCS-35S promoter was not found to be better than the 35S promoter. Vector pDPG351 gave significantly higher levels of transient expression than pDPG350, but not higher than pDPG215.

For independent stable transformants, a wide range of luciferase expression has been observed in experiments using either pDPG215 or pDPG315. For both plasmids luciferase expression as measured in callus using the scintillation counter ranged between 100 and  $2 \times 10^6$  CPM. Luciferase expression has been confirmed in  $R_0$  plants.

Microspore-derived cell clusters (genotype G238) were bombarded with the p350 and p351 constructs. This tissue was grown on non-selective 227 medium, and was screened 2-3 weeks post-bombardment using the multiwell luminometer.

Out of six plates screened, two wells produced readings potentially above background. This tissue was transferred to fresh 227. Also, AT824 suspension samples were bombarded with the p350 and p351. After 3 days on the filter, the tissue was put back into liquid and screening for luciferase activity was started 12 days post-bombardment.

**Example 28:      Extension: A Secreted Screenable Marker**

Initially, candidates considered as screenable markers have been genes encoding intracellular proteins that require diffusible substrates or permeation of cells to perform the assay. An alternative is a secretable marker. Three general types have been considered: (i) functional secreted enzymes detectable by assaying catalytic activity, (ii) small diffusible proteins detectable by ELISA, such as IL-2, or (iii) secreted markers that remain sequestered in the cell wall that also contain unique epitopes for antibody detection.

Candidates for functional secreted markers that could be detected through catalytic action would include such enzymes as  $\beta$ -galactosidase and  $\beta$ -glucuronidase. Unfortunately, these enzymes are modified during the secretion process in a manner which renders them inactive. For example, GUS enters the endoplasmic reticulum and is N-glycosylated which blocks enzyme activity (Iturraga *et al.*, 1989). Recently, the N-linked glycosylation site was altered by site-directed mutagenesis (Farrell & Beachy, 1990), but no reports on secretion and/or functional activity have yet followed. In using GUS, it is not clear whether the colorimetric product would remain localized to the point where clear demarcation of expressing and non-expressing cells could be achieved, but this is still a promising marker.

A number of small proteins such as interleukins have been well characterized in terms of molecular genetics and immunodetection. However, even if properly targeted across the plasma membrane, the best candidates are all small enough to

diffuse readily through the cell wall into the extracellular solution. Thus, they could be detected by ELISA methods, but could not be localized to specific cells. A variety of mammalian genes are known that would provide a unique epitope for labeling. However, large mammalian proteins secreted across the plasma  
5 membrane would not be likely to reach the surface of the wall (and hence be relatively inaccessible), while small proteins would diffuse into the extracellular space.

The requirements of a secreted antigen construct were thus determined to  
10 be: encoding a unique epitope sequence that would provide low background in plant tissue; a promoter-leader sequence that would impart efficient expression and targeting across the plasma membrane; and the production of a protein which is bound in the cell wall and yet accessible to antibodies. The expression of a modified, but otherwise normally-secreted, cell wall constituent was considered to  
15 be an ideal candidate for a secretable marker. Extension, HPRG, was the cell wall protein chosen since this molecule is fairly well characterized in terms of molecular biology, expression and protein structure, and the maize genomic HPRG sequence was available (from Dr. Pedro Puigdomenech).

The strategy for visualizing the expression of the introduced extension gene  
20 revolves around introducing a novel epitope into the secreted protein, which could then be localized using immunological techniques. Certain preliminary tests need to be performed before making such a construct. A novel epitope must be identified for which a high-titer antibody is available; maize extracts should be reacted with  
25 the antibody to ensure there is no non-specific background labeling; and the feasibility of immunolabeling the cell wall of living cells should be determined.

The epitope chosen was a 15 amino acid sequence from the pro-region of  
B murine interleukin-1- $\beta$ , MATVPELNCEMPPSD (seq id no:1), which was recognized  
30 by polyclonal antibodies. A dot-blot was performed loading either 0, 10, or 50 ng of a 31 kd recombinant protein, and testing with either R1682 serum, normal rabbit

serum, Fc-purified R1684, or Fc-purified normal rabbit. With the higher protein load (50 ng), background was observed in the normal rabbit and the Fc-purified normal rabbit at the higher antibody concentrations (i.e. 1:300, and 1:100). No background was observed in the 10 ng dots at antibody dilutions greater (less concentrated) than 1:100. For the R1682 serum, protein was detected for both the 10 and 50 ng dots at all antibody dilutions, even down to 1:3000. The Fc-purified R1684 was approximately 10-fold less sensitive. This result indicates that the R1682 antibody is very high-titer, and should be useful as a marker system.

On analyzing the IL-1- $\beta$  pro sequence in computer gene and protein data banks, no sequence homology was found in either plants or fungi. Testing extracts from maize suspension cells and from cell walls confirmed that no background labeling exists.

Using colloidal gold conjugated secondary antibody followed by silver enhancement, surface labeling of "living" root tips was verified. The labeling of the surface with both the primary and secondary antibodies was performed under physiological conditions (low concentrations of organic buffers and/or salts). However, in order to visualize the gold label a silver enhancement process was then utilized, and this is toxic. The use of a new fluorophore, phycoerythrin, conjugated to the secondary antibody is also contemplated. This should eliminate problems with endogenous background fluorescence and increase resolution.

The necessary oligonucleotides were made and a cloning strategy was developed for inserting the novel IL-1 sequence into the carboxyl-end of the extension structural gene and placing this into a plant expression vector (CaMV 35S promoter, *Agrobacterium tumefaciens* transcript 7' 3' region).

## K. Co-Transformation

Co-transformation may be achieved using a vector containing the marker and another gene or genes of interest. Alternatively, different vectors, e.g., plasmids, may contain the different genes of interest, and the plasmids may be concurrently delivered to the recipient cells. Using this method, the assumption is made that a certain percentage of cells in which the marker has been introduced, have also received the other gene(s) of interest. As can be seen in the following examples, not all cells selected by means of the marker, will express the other genes of interest which had been presented to the cells concurrently. For instance, in Example 29, successful cotransformation occurred in 17/20 independent transformants (see Table 5), coexpression occurred in 4/20. In some transformants, there was variable expression among transformed cells.

### **Example 29: Co-Integration and Co-Expression of the Bar Gene and the GUS Gene to Cell Lines Derived from the SC82 Suspension Culture**

Of the bialaphos-resistant isolates selected from a reinitiation of cryopreserved SC82 cells transformed with separate plasmids (as described for SC716), nineteen independent transformants were selected in this experiment (experiment #6, Table 5). The frequency of cointegration and coexpression in those isolates was similar to that described for SC716 isolates (Table 5). The pattern of GUS staining in these transformants varied in a manner similar to that described for coexpressing SC716 transformants. A transformant, Y13, which contained intact GUS coding sequence, exhibited varying levels of GUS activity as shown in Figure 6. This type of expression pattern has been described previously in cotransformed BMS cells (Klein *et al.*, 1989). Variable activity detected in the cells from a single transformant may be attributed to unequal penetration of the GUS substrate, or differential expression, methylation, or the absence of the gene in some cells.

These results show that both the *bar* gene and the GUS gene are present in some of the cells bombarded with the two plasmids containing these genes. Co-transformation has occurred. In the cotransformation examples described herein and summarized in Table 5, cotransformation frequency of the non-selected gene was 77%; coexpression frequency was 18%.

#### L. Regeneration of Plants From Transformed Cells

For use in agriculture, transformation of cells *in vitro* is only one step toward commercial utilization of these new methods. Plants must be regenerated from the transformed cells, and the regenerated plants must be developed into full plants capable of growing crops in open fields. For this purpose, fertile corn plants are required. The invention disclosed herein is the first successful production of fertile maize plants (e.g., see Figure 7A) from transformed cells.

During suspension culture development, small cell aggregates (10-100 cells) are formed, apparently from larger cell clusters, giving the culture a dispersed appearance. Upon plating these cells to solid media, somatic embryo development can be induced, and these embryos can be matured, germinated and grown into fertile seed-bearing plants. The characteristics of embryogenicity, regenerability, and plant fertility are gradually lost as a function of time in suspension culture. Cryopreservation of suspension cells arrests development of the culture and prevents loss of these characteristics during the cryopreservation period.

#### **Example 30: Regeneration of Plants from SC82 and SC716**

One efficient regeneration system involves transfer of embryogenic callus to MS (Murashige & Skoog, 1962) medium containing 0.25 mg/l 2,4-dichlorophenoxyacetic acid and 10.0 mg/l 6-benzyl-aminopurine. Tissue was maintained on this medium for approximately 2 weeks and subsequently transferred to MS medium without growth regulators (Shillito *et al.*, 1989). Shoots



that developed after 2-4 weeks on growth regulator-free medium were transferred to MS medium containing 1% sucrose and solidified with 2 g/l Gelgro<sup>R</sup> in Plant Con<sup>R</sup> containers where rooting occurred.

5            Another successful regeneration scheme involved transfer of embryogenic callus to N6 (Chu *et al.*, 1975) medium containing 6% sucrose and no growth regulators (Armstrong & Green, 1985) for two weeks followed by transfer to MS medium without growth regulators as described above. Regeneration was performed at 25°C under fluorescent lights (250 microeinsteins·m<sup>-2</sup>·s<sup>-1</sup>). After  
10        approximately 2 weeks developing plantlets were transferred to a Plant Con<sup>®</sup> container containing medium 501. When plantlets had developed 3 leaves and 2-3 roots they were transferred to soil, hardened off in a growth chamber (85% relative humidity, 600 ppm CO<sub>2</sub>, 250 microeinsteins·m<sup>-2</sup>·s<sup>-1</sup>), and grown to maturity either in a growth chamber or the greenhouse.

15            Regeneration of plants from transformed cells requires careful attention to details of tissue culture techniques. One of the major factors is the choice of tissue culture media. There are many media which will support growth of plant cells in suspension cultures, but some media give better growth than others at  
20        different stages of development. Moreover, different cell lines respond to specific media in different ways. A further complication is that treatment of cells from callus initiation through transformation and ultimately to the greenhouse as plants, requires a multivariate approach. A progression consisting of various media types, representing sequential use of different media, is needed to optimize the proportion  
25        of transformed plants that result from each cell line. Table 6 illustrates one sequential application of combinations of tissue culture media to cells at different stages of development. Successful progress is ascertained by the total number of plants regenerated.

30            It can be seen that using the same group of media, cell lines will vary in their success rates (number of plants) (Table 6). There was also variation in overall

success rate, line AO1-15 yielding the greatest number of plants overall. (It should be noted, however, that because tissue was limiting not all combinations of media were used on all lines, therefore, overall comparisons are limited.)

5           A preferred embodiment for use on cell lines SC82 and SC716, at least initially, is the combination shown in the second column under the regeneration media progression (media 227, 171, 101, 501). Media 227 is a good media for the selective part of the experiments, for example, to use for growth of callus in the presence of bialaphos. This media contains the growth regulator dicamba.  
10       NAA and 2,4-D are growth regulators in other media. In liquid media, these may be encapsulated for controlled release (Adams, W. et al., in preparation).

          Thus, it can be seen from Table 1 that the various media are modified so as to make them particularly applicable to the development of the transformed plant at the various stages of the transformation process. For example, subculture of cells  
15       in media 171 after applying the selective agent, yields very small embryos. Moreover, it is believed that the presence of BAP in the media facilitates development of shoots. Myo-inositol is believed to be useful in cell wall synthesis. Shoot elongation and root development proceeds after transfer to media 101. 101  
20       and 501 do not contain the growth regulators that are required for earlier stages of regeneration.

**Table 6: Plants to Soil From Bombardment of SC716 (Expts 1,2; Table 6):**

## REGENERATION MEDIA PROGRESSIONS

[illegible]

**X** = Regeneration not attempted by this route.  
**\*** = More plants could have been taken to soil.

**201b = 201 with 1 mg/1 bialophos.**  
**227b = 227 with 1 mg/1 bialophos.**

Transfer of regenerating plants is preferably completed in an agar-solidified media adapted from a nutrient solution developed by Clark (1982), media 501. The composition of this media facilitates the hardening of the developing plants so that they can be transferred to the greenhouse for final growth as a plant. The salt concentration of this media is significantly different from that of the three media used in the earlier stages, forcing the plant to develop its own metabolic pathways. These steps toward independent growth are required before plants can be transferred from tissue culture vessels (e.g. petri dishes, plant cans) to the greenhouse.

Approximately 50% of transformed callus lines derived from the initial SC82 and SC716 experiments were regenerable by the routes tested. Transgenic plants were regenerated from four of seven independent SC82 transformants and ten of twenty independent SC716 transformants.

Regeneration of thirteen independently, transformed cell lines and two control lines of SC716 was pursued. Regeneration was successful from ten of thirteen transformants. Although a total of 458 plantlets were regenerated, due to time and space constraints only 219 transformed plants (representing approximately 48% of the total number of regenerants) were transferred to a soilless mix (see below). Approximately 185 plants survived. Twelve regeneration protocols were investigated and the number of plants regenerated from each route has been quantified (Table 6). There appeared to be no significant advantage to maturing the tissues on 201, 52, 163, or 205 (see Table 1 for media codes) prior to transfer to medium 171 or 173. The majority of the plants were generated by subculturing embryogenic callus directly from 227 to either 171 or 173. These plantlets developed roots without addition of exogenous auxins, and plantlets were then transferred to a soilless mix, as was necessary for many of the transformants regenerated from SC82.

The soilless mix employed comprised Pro Mix, Micromax, Osmocote 14-14-14 and vermiculite. Pro Mix is a commercial product used to increase fertility and porosity as well as reduce the weight of the mixture. This is the bulk material in the mixture. Osmocote is another commercial product that is a slow release  
5 fertilizer with a nitrogen-phosphorus-potassium ratio of 14:14:14. Micromax is another commercial fertilizer that contains all of the essential micronutrients. The ratio used to prepare the soilless mix was: 3 bales (3 ft<sup>3</sup> each) Pro Mix; 10 gallons (vol.) vermiculite; 7 pounds Osmocote; 46 ml Micromax. The soilless mix may be  
10 supplemented with one or two applications of soluble Fe to reduce interveinal chlorosis during early seedling and plant growth.

Regeneration of transformed SC82 selected cell lines yielded 76 plants transferred to the soilless mix, and 73 survived. The plants were regenerated from six bialaphos-resistant isolates, representing four of seven clonally independent  
15 transformants. Eighteen protocols were used successfully to regenerate the seventy six plants (Table 7). Differences in morphology between cell lines deemed some protocols more suitable than others for regeneration.

## TABLE 7

# EFFECTS OF PROGRESSION OF MEDIA ON THE NUMBER OF PLANTS REGENERATED (SC82)\*

[illegible]

**\* = See table 1 for media codes.**

**X = This media progression was either attempted and unsuccessful or not attempted.**

227A = 227 with  $10^{-7}$  M ABA.

**227B = 227 with 1 mg/1 bialaphos.**

Prior to regeneration, the callus was transferred to either a) an N6-based medium containing either dicamba or 2,4-D or b) an MS-based medium containing 2,4-D. These steps allowed further embryoid development prior to maturation. Most of the maturation media contained high BAP levels (5-10mg/l) to enhance shoot development and cause proliferation. An MS-based medium with low 2,4-D (0.25 mg/l) and high BAP (10 mg/l), as described by Shillito *et al.*, 1989, was found to be quite effective for regeneration.

Likewise, an MS-based medium containing 1 $\mu$ m NAA, 1  $\mu$ m IAA, 2  $\mu$ m 2-IP, and 5 mg/l BAP (modified from Conger *et al.*, 1987) also promoted plant regeneration of these transformants. After plantlets recovered by any of the regenerative protocols had grown to five cm, they were transferred to a nutrient solution described by Clark, 1982, supplemented with 2% sucrose and solidified with Gelgro. Plantlets which were slow to develop roots were treated with 3  $\mu$ l droplets of 0.3% IBA at the base of the shoot to stimulate rooting. Plants with well developed root systems were transferred to a soilless mix and grown in controlled environmental chambers from 5-10 days, prior to transfer to the greenhouse.

### **Example 31: Regeneration of AT824 Transformants**

Transformants were produced as described in examples 19 and 20. For regeneration tissue was first transferred to solid medium 223 and incubated for two weeks. Transformants may be initially subcultured on any solid culture that supports callus growth, e.g., 223, 425, 409 and so forth. Subsequently transformants were subcultured one to three times, but usually twice on 189 medium (first passage in the dark and second passage in low light) and once or twice on 101 medium in petri dishes before being transferred to 607 medium in Plant Cons<sup>®</sup>. Variations in the regeneration protocol are normal based on the progress of plant regeneration. Hence some of the transformants were first

subcultured once on 425 medium, twice on 189 medium, once or twice on 101 medium followed by transfer to 501 medium in Plant Cons®. As shoots developed on 101 medium, the light intensity was increased by slowly adjusting the distance of the plates from the light source located overhead. All subculture intervals were for about 2 weeks at 24°C. Transformants that developed 3 shoots and 2-3 roots were transferred to soil.

Plantlets in soil were incubated in an illuminated growth chamber and conditions were slowly adjusted to adapt or condition the plantlets to the drier and more illuminated conditions of the greenhouse. After adaptation/conditioning in the growth chamber, plants were transplanted individually to 5 gallon pots of soil in the greenhouse.

#### **M. Assays for Integration of Exogenous DNA and Expression of DNA in R<sub>0</sub>-R<sub>1</sub> Plants**

Studies were undertaken to determine the expression of the transformed gene(s) in transgenic R<sub>0</sub> and R<sub>1</sub> plants. Functional activity of PAT was assessed by localized application of a commercial herbicide formulation containing PPT to leaves of SC82 R<sub>0</sub> and R<sub>1</sub> plants. No necrosis was observed on leaves of R<sub>0</sub> plants containing either high levels (E2/E5), or low levels (E3/E4) of PAT. Herbicide-treated E3/E4/E6 and control leaves are shown in Figure 8A. Herbicide was also applied to leaves of E2/E5 progeny segregating for *bar*. As demonstrated in Figure 8B, leaves of R<sub>1</sub> plants expressing *bar* exhibited no necrosis six days after application of the herbicide while R<sub>1</sub> plants without *bar* developed necrotic lesions. No necrosis was observed on transformed leaves up to 30 days post-application.

Twenty-one R<sub>0</sub> plants, representing each of the four regenerable transformed SC82 callus lines, were also analyzed for expression of the *bar* gene product, PAT,



by thin-layer chromatographic techniques. Protein extracts from the leaves of the plants were tested. PAT activity of one plant regenerated from each callus line is shown in Fig. 9.

5 All 21 plants tested contained PAT activity. Furthermore, activity levels were comparable to levels in the callus lines from which the plants were regenerated. The nontransformed plant showed no PAT activity (no band is in the expected position for acetylated PPT in the autoradiograph from the PAT chromatogram). A band appears in the BMS lane that is not in lanes containing  
10 protein extracts from the plant leaves. This extra band was believed to be an artifact.

As another method of confirming that genes had been delivered to cells and integrated, genomic (chromosomal) DNA was isolated from a nontransformed plant,  
15 the four regenerable callus lines and from two R<sub>0</sub> plants derived from each callus line. Figure 10 illustrates results of gel blot analysis of genomic DNA from the four transformed calli (C) and the R<sub>0</sub> plants derived from them. The transformed callus and all plants regenerated from transformed callus contained sequences that hybridized to the *bar* probe, indicating the presence of DNA sequences that were  
20 complementary to *bar*. Furthermore, in all instances, hybridization patterns observed in plant DNA were identical in pattern and intensity to the hybridization profiles of the corresponding callus DNA.

DNA from E3/E4/E6 callus and the desired R<sub>0</sub> plants contained  
25 approximately twenty intact copies of the 1.9 kb *bar* expression unit (Cauliflower Mosaic Virus 35S promoter-*bar*-Agrobacterium transcript 7' 3'-end) as well as numerous other *bar*-hybridizing fragments. E11 callus and plant DNA contained 1-2 copies of the intact expression unit and 5-6 additional non-intact hybridizing fragments. E10 callus and plants contained 1-2 copies of the intact *bar* expression  
30 unit. E2/E5 DNA contained a single fragment of approximately 3 kb that hybridized

to the probe. To confirm that the hybridizing sequence observed in all plants were integrated into the chromosomal DNA, undigested genomic DNA from one plant derived from each independent transformant was analyzed by DNA gel blot hybridization. Hybridization to *bar* was observed only in high molecular weight DNA providing evidence for the integration of *bar* into the maize genome.

Plants were regenerated from the coexpressing callus line, Y13, shown in Figure 6. Plants regenerated from Y13 (experiment #6, Table 5) were assayed for GUS activity and histochemically stained leaf tissue from one plant is shown in Figures 8C, D, E. Numerous cell types including epidermal, guard, mesophyll and bundle sheath cells stained positive for GUS activity. Staining intensity was greatest in the vascular bundles. Although all leaf samples from the regenerated plants tested (5/5) expressed the nonselected gene, some non-expressing leaf sectors were also observed. Leaf tissue extracts from three Y13 and three control plants were also assayed for GUS activity by fluorometric analysis (Jefferson, 1987). Activity detected in two opposing leaves from each of three Y13 plants tested was at least 100-fold higher than that in control leaves.

### Example 32: General Methods for Assays

A method to detect the presence of phosphinothricin acetyl transferase (PAT) activity is to use an *in vitro* enzyme reaction followed by thin layer chromatography.

An example of such detection is shown in Fig. 9 wherein various protein extracts prepared from homogenates of potentially transformed cells, and from control cells that have neither been transformed nor exposed to bialaphos selection, are assayed by incubation with PPT and <sup>14</sup>C-Acetyl Coenzyme A followed by thin layer chromatography. 25 µg of protein extract were loaded per lane. The source in lanes E1-E11 were SC82 transformants; B13 is a BMS (Black

Mexican Sweet corn nonembryogenic) *bar* transformant. E0 is a nonselected, nontransformed control.

As can be seen at the position indicated by the arrow (the position expected for the mobility of  $^{14}\text{C}$ -N-AcPPT), all lanes except the nontransformed control exhibit PAT activity by the formation of a compound with the appropriate mobility expected for  $^{14}\text{C}$ -N-Acetyl PPT. Variation in activity levels among the transformants was approximately 10 fold, as demonstrated by the relative intensity of the bands. The results of this assay provide confirmation of the expression of the *bar* gene which codes for PAT. For analysis of PAT activity in plant tissue, 100-200 mg of leaf tissue was extracted in sintered glass homogenizers and assayed as described previously.

GUS activity was assessed histochemically as described using 5-bromo-4-chloro-3-indolyl glucuronide (Jefferson, 1987); tissue was scored for blue cells 18-24 h after addition of substrate. Fluorometric analysis was performed as described by Jefferson (1987) using 4-methyl umbelliferyl glucuronide.

DNA analysis was performed as follows. Genomic DNA was isolated using a procedure modified from Shure, *et al.*, 1983. Approximately 1 gm callus tissue was ground to a fine powder in liquid N<sub>2</sub> using a mortar and pestle. Powdered tissue was mixed thoroughly with 4 ml extraction buffer (7.0 M urea, 0.35 M NaCl, 0.05 M Tris-HCl pH 8.0, 0.01 M EDTA, 1% sarcosine). Tissue/buffer homogenate was extracted with 4 ml phenol/ chloroform. The aqueous phase was separated by centrifugation, passed through Miracloth, and precipitated twice using 1/10 volume of 4.4 M ammonium acetate, pH 5.2 and an equal volume of isopropanol. The precipitate was washed with 70% ethanol and resuspended in 200-500  $\mu\text{l}$  TE (0.01 M Tris-HCl, 0.001 M EDTA, pH 8.0). Plant tissue may also be employed for the isolation of DNA using the foregoing procedure.

The presence of a gene in a transformed cell may be detected through the use of polymerase chain reaction (PCR). Using this technique specific fragments of DNA can be amplified and detected following agarose gel electrophoresis. For example the *bar* gene may be detected using PCR. Two hundred to 1000 ng genomic DNA is added to a reaction mix containing 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.1 mg/ml gelatin, 200 uM each dATP, dCTP, dGTP, dTTP, 0.5 uM each forward and reverse DNA primers, 20% glycerol, and 2.5 units Taq DNA polymerase. The forward primer is CATCGAGACAAGCACGGTCAACTTC. The reverse primer is AAGTCCCTGGAGGCACAGGGCTTCAAGA. PCR amplification of *bar* using these primers requires the presence of glycerol, but this component is not needed for most other applications. The reaction is run in a thermal cycling machine as follows: 3 minutes at 94C, 39 repeats of the cycle 1 minute at 94C, 1 minute at 50C, 30 seconds at 72C, followed by 5 minutes at 72C. Twenty ul of each reaction mix is run on a 3.5% NuSieve gel in TBE buffer (90 mM Tris-borate, 2 mM EDTA) at 50V for two to four hours. Using these primers a 279 base pair fragment of the *bar* gene is amplified.

For Southern blot analysis genomic DNA was digested with a 3-fold excess of restriction enzymes, electrophoresed through 0.8% agarose (FMC), and transferred (Southern, 1975) to Nytran (Schleicher and Schuell) using 10X SCP (20X SCP: 2 M NaCl, 0.6 M disodium phosphate, 0.02 M disodium EDTA). Filters were prehybridized at 65°C in 6X SCP, 10% dextran sulfate, 2% sarcosine, and 500 µg/ml heparin (Chomet *et al.*, 1987) for 15 min. Filters were hybridized overnight at 65°C in 6X SCP containing 100 µg/ml denatured salmon sperm DNA and <sup>32</sup>P-labeled probe. The 0.6 kb SmaI fragment from pDPG165 and the 1.8 kb BamHI/EcoRI fragment from pCEV5 were used in random priming reactions (Feinberg & Vogelstein, 1983; Boehringer-Mannheim) to generate labeled probes for detecting sequences encoding PAT or GUS, respectively. Filters were washed in 2X SCP, 1% SDS at 65°C for 30 min. and visualized by autoradiography using Kodak XAR5 film. Prior to rehybridization with a second probe, the filters were

boiled for 10 min. in distilled H<sub>2</sub>O to remove the first probe and then prehybridized as described above.

#### N. Fertility of Transgenic Plants

5

10

To recover progeny the regenerated, genetically transformed maize plants (designated R<sub>0</sub>), were backcrossed with pollen collected from nontransformed plants derived from seeds. Alternatively pollen was collected from R<sub>0</sub> plants and used to pollinate nontransformed plants. Progeny (designated R<sub>1</sub>) that contained and expressed *bar* were recovered from crosses in which the transformant was used as a male or female parent.

15

An important aspect of this invention is the production for the first time of fertile, genetically transformed maize plants (R<sub>0</sub>) and progeny (R<sub>1</sub>). These were regenerated from embryogenic cells that were transformed. R<sub>1</sub> plants are those resulting from backcrossing of R<sub>0</sub> plants.

20

25

Pollination of transgenic R<sub>0</sub> ears with non-transformed B73 pollen resulted in kernel development. In addition, kernels developed from pistillate flowers on male inflorescences that were pollinated with non-transformed B73 pollen. Kernels on transformed R<sub>0</sub> plants from SC82 developed normally for approximately 10-14 days post-pollination but after this period the kernels ceased development and often collapsed. Most plants exhibited premature senescence at this time. A total of 153 kernels developed sporadically on numerous plants (see Table 8): 8 of 37 E2/E5 plants, 2 of 22 E10 plants, and 3 of 6 E11 plants. Viable progeny were recovered by embryo rescue from 11 E2/E5 plants and one E10 plant.

30

SC716 R<sub>0</sub> plants were also backcrossed with seed-derived B73 plants. To date, from the 35 mature SC716 R<sub>0</sub> plants nine plants (representing four independent callus lines) yielded 51 kernels, 31 of which produced vigorous R<sub>1</sub>

seedlings (Table 8). Most kernels that developed on SC716 plants did not require embryo rescue. Kernels often developed for 30-40 days on the plant and some were germinated in soil. The remaining seed was germinated on MS-based medium to monitor germination and transferred to soil after a few days. In addition to the improved kernel development observed on SC716 R<sub>0</sub> plants relative to SC82 R<sub>0</sub> plants, pollen dehiscence from anthers of several SC716 plants and some of this pollen germinated *in vitro* (Pfahler, 1967). Transmission of the foreign gene has occurred both through SC716 R<sub>1</sub> ears and using SC716 R<sub>1</sub>-derived pollen on non-transformed ears.

Table 8. Regenerated Plants ( $R_o$ ) and Progeny ( $R_1$ )

Exp. #	Culture Bombarded	# of Independent bar Transformants Recovered	# of Regenerable Transformed Callus Lines	# of $R_o$ Plants	# Reaching Maturity	# of $R_o$ Producing Kernels	# of Kernels Recovered	# of $R_1$ Plants
5								
1, 2	SC82	7	4	76	73	23	153	40
10								
4, 5	SC716	20	10	219	(35)	(9)	(51)	(31)
3	SC94	8	2 <sup>a</sup>	11 <sup>a</sup>	(0)	(0)	(0)	(0)
6	SC82	19	4 <sup>a</sup>	23 <sup>a</sup>	(0)	(0)	(0)	(0)

15

<sup>a</sup> Regeneration in progress.

() Experiment still in progress, data still being collected.

To date fertile plants from 267 transgenic lines have produced over 59, 577 seed (about 227 R<sub>1</sub> seed per transgenic line). Table 2 indicates that these plants were derived from 11 different cell lines. In addition both male and female fertility has been observed in many of these cells lines. Kernels routinely mature on plants for which the transformant is either the male or the female parent. Embryo rescue is only necessary under unusual circumstances.

Pollen obtained from transformed R<sub>1</sub> plants has been successfully employed to pollinate B73 ears and a large number of seeds have been recovered (see Figure 7C). Moreover, a transformed ear from an R<sub>1</sub> plant crossed with pollen from a non-transformed inbred plant is shown in Figure 7D. The fertility characteristics of the R<sub>1</sub> generation has been confirmed both from a standpoint of the pollen's ability to fertilize non-transformed ears, and the ability of R<sub>1</sub> ears to be fertilized by pollen from non-transformed plants. Fertility of transgenic plants has been maintained for at least 12 generations.

By providing fertile, transgenic offspring, the practice of the invention allows one to subsequently, through a series of breeding manipulations, move a selected gene from one corn line into an entirely different corn line without the need for further recombinant manipulation. Movement of genes between corn lines is a basic tenet of the corn breeding industry, involving simply backcrossing the corn line having the desired gene (trait). Introduced transgenes are valuable in that they behave genetically as any other corn gene and can be manipulated by breeding techniques in a manner identical to any other corn gene. Exemplary procedures of this nature have been successfully carried out by the inventors. In these backcrossing studies, the gene for resistance to the herbicide Basta®, *bar*, has been moved from two transformants derived from cell line SC716 and one transformant derived from cell line SC82 into 18 elite inbred lines by backcrossing. It is possible from these 18 inbreds to make a large number of hybrids of commercial importance. Eleven of the possible hybrids have been made and are being field



tested for yield and other agronomic characteristics and herbicide tolerance. Additional backcrossing to a further 68 elite inbred lines is underway.

**Example 33:      Analysis of Progeny ( $R_1$ ) of**  
**Transformed  $R_0$  Plants for PAT and *bar***

A total of 40 progeny of E2/E5  $R_0$  plants were analyzed for PAT activity, ten of which are shown in Figure 11A. Of 36 progeny which were assayed, 18 had PAT activity. Genomic DNA from the same ten progeny analyzed for PAT activity was analyzed by DNA gel blot hybridization for the presence of *bar* as shown in Figure 11B. The six progeny tested that expressed PAT contained a single copy of *bar* identical in mobility to that detected in callus and  $R_0$  plants; the four PAT-negative progeny tested did not contain *bar*-hybridizing sequences. In one series of assays, the presence of the *bar* gene product in 18 of 36 progeny indicates a 1:1 segregation of the single copy of *bar* found in E2/E5  $R_0$  plants and is consistent with inheritance of PAT expression as a single dominant trait. A dominant pattern of inheritance would indicate the presence in the plant of at least one copy of the gene coding for PAT. The single progeny recovered from an E10  $R_0$  plant tested positive for PAT activity.

It was determined that the methods disclosed in this invention resulted in transformed  $R_0$  and  $R_1$  plants that produced functionally active PAT. This was determined by applying Basta (PPT) to the leaves of plants and determining whether necrosis (tissue destruction) resulted from this application. If functionally active PAT is produced by the plants, the leaf tissue is protected from necrosis. No necrosis was observed on  $R_0$  plants expressing high levels of PAT (E2/E5) or on plants expressing low levels (E3/E4/E6) (Fig. 8A).

Herbicide was also applied to leaves of  $R_1$  progeny segregating for *bar*. In these studies, no necrosis was observed on  $R_1$  plants containing and expressing

*bar*, however, necrosis was observed on those R<sub>1</sub> plants lacking the *bar* gene. This is shown in Fig 8B.

Segregation of *bar* did not correlate with the variability in phenotypic characteristics of R<sub>1</sub> plants such as plant height and tassel morphology. In Figure 5B, the plant on the right contains *bar*, the plant on the left does not. In addition, most plants were more vigorous than the R<sub>0</sub> plants.

Of the 23 R<sub>1</sub> seedlings recovered in this experiment from the SC716 transformants, ten of 16 had PAT activity. PAT activity was detected in four of ten progeny from R<sub>0</sub> plants representing callus line R18 and six of six progeny from R<sub>0</sub> plants representing callus line R9.

#### O. Embryo Rescue

In cases where embryo rescue was required, developing embryos were excised from surface disinfected kernels 10-20 days post-pollination and cultured on medium containing MS salts, 2% sucrose and 5.5 g/l Seakem agarose. Large embryos (> 3 mm) were germinated directly on the medium described above. Smaller embryos were cultured for approximately 1 week on the above medium containing 10<sup>-5</sup>M abscisic acid and transferred to growth regulator-free medium for germination. Embryos that became bacterially contaminated; these embryos were transferred to medium containing 300 µg/ml cefoxitin. Developing plants were subsequently handled as described for regeneration of R<sub>0</sub> plants.

**Example 34:      Embryo Rescue**

Viable progeny, recovered from seven SC82 E2/E5 plants and one SC82 E10 plant, were sustained by embryo rescue. This method consisted of excising embryos from kernels that developed on R<sub>0</sub> plants. Embryos ranged in size from about 0.5 to 4 mm in length. Small embryos were cultured on maturation medium containing abscisic acid while larger embryos were cultured directly on germination medium. Two of the approximately forty viable progeny recovered from SC82 R<sub>0</sub> plants by embryo rescue are shown in Figure 7B.

**P.      Phenotype of Transgenic Plants**

Most of the R<sub>0</sub> plants regenerated from SC82 transformants exhibited an A188 X B73 hybrid phenotype. Plants were similar in height to seed derived A188 plants (3-5 feet) but had B73 traits such as anthocyanin accumulation in stalks and prop roots, and the presence of upright leaves. Many plants, regardless of the callus line from which they were regenerated, exhibited phenotypic abnormalities including leaf splitting, forked leaves, multiple ears per node, and coarse silks. Although many of the phenotypic characteristics were common to all R<sub>0</sub> plants, some characteristics were unique to plants regenerated from specific callus lines. Such characteristics were exhibited regardless of regeneration route and the time spent in culture during regeneration.

Nontransformed control plants were not regenerated from this culture and, therefore, cannot be compared phenotypically. Pistillate flowers developed on tassels of one E11 (1/6), several E10 (3/22) and almost one-third of the E2/E5 (12/37) plants with a range of three to approximately twenty ovules per tassel. Primary and secondary ears developed frequently on most E2/E5, E10, and E11 plants; a mature E2/E5 plant is shown in Figure 7A. Anthers rarely extruded from the tassels of plants regenerated from SC82 transformants and the limited number

of anthers which were extruded did not dehisce pollen. Some phenotypic characteristics observed were unique to plants regenerated from a specific callus line such as the lack of ears on E3/E4/E6 plants and a "grassy" phenotype (up to 21 long narrow leaves) exhibited by all E11 plants.

5

All SC82 plants senesced prematurely; leaf necrosis began approximately two weeks after anthesis. The  $R_0$  plants regenerated from SC82 transformed cell lines have tended to senesce prematurely; typically before the developing kernels were mature. This has necessitated the use of embryo rescue to recover progeny (10  $R_1$  generation). Segregation of *bar* in the  $R_1$  generation does not correlate with the variability in phenotypic characteristics of  $R_1$  plants such as plant height and tassel morphology. In Figure 7B, the plant on the right contains *bar*, the plant on the left does not. In addition, most of the  $R_1$  plants are more vigorous than the  $R_0$  plants. Transformed progeny ( $R_1$ ) have produced kernels and progeny testing has (15 now been advanced to the  $R_{12}$  generation.

Of 219 plants regenerated from 10 independent SC716 transformants, approximately 35 reached maturity (Table 8). The SC716 plants did not exhibit the phenotypic differences which characterized the plants regenerated from the (20 individual callus lines of SC82. These plants were more uniform and abnormalities less frequent. The phenotype of these plants closely resembled that of control plants regenerated from a SC716 cryopreserved culture which was not bombarded. Plant height ranged from three to six feet with the majority of the plants between five and six feet. Most mature plants produced large, multi-branched tassels and (25 primary and secondary ears. Pistillate flowers also developed on tassels of several SC716 plants. Although anther extrusion occurred at approximately the same low frequency as in the SC82 plants, a small amount of pollen dehiscence from some extruded anthers. For most of the SC716 plants that reached maturity, senescence did not commence until at least 30 days after anthesis.

(30 The improved characteristics of SC716 plants over SC82 plants indicate that

differences between the suspension cultures may be responsible. This observation has been supported by further experiments in which AT824 plants have been regenerated. These plants are normal in appearance. Plants produce normal tassels and shed viable pollen. In addition plants do not prematurely senesce and seed will mature on the plant. Many plants derived from this cell line and the lines ABT4 and Hi-II are indistinguishable from nontransformed plants.

#### I. Transformation with Genes for Desirable Traits

One of the distinct advantages provided by the present invention is the ability to transform monocot plants, such as maize, with a gene or genes which imparts a desirable trait to the resultant transgenic plants. These traits include, for example, resistance to insects, herbicides, drought, etc., and the improvement of characteristics such as appearance, yield, nutritional quality, and the like. Certain such genes which are highly desirable in monocot transformation have been discussed as selectable markers, for example, *bar* and EPSPS. These genes encode proteins which confer herbicide resistance on the plant. Other particularly preferred transgenes include those that have insecticidal activities, such as toxins, proteinase inhibitors and lectins, and those genes that alter the nutritional quality of the grain. The following examples illustrate the use of the present invention in generating advantageous transgenic plants. Table 9 lists all of the genes successfully introduced into maize by the inventors and summarizes the status of analysis for the presence of the introduced DNA and expression. The Table indicates that stable transformants have been recovered containing all genes attempted. Expression has been detected from all structural genes listed in at least one transformed cell line in studies that have progressed to this stage. Detection of expression is dependent on the promoter and enhancers used to drive expression of the structural gene, the structural gene itself, and the limits of the detection system. At this point in time fertile plants containing the *uidA*, *bar*, *Bt*, *aroA*, *dapA*, 10 kD zein storage protein and

hygromycin resistance genes have been recovered from transformants. Expression of the *bar* gene has been detected in progeny from all transformants examined (19/19). Expression of the *uidA* gene has been detected in the progeny of one out of five transformants assayed.

5

The protocols employed for preparing the transgenic plants described in the foregoing Table were as described above. The preparation of the various vectors, *etc.*, was accomplished through the application of molecular biology techniques as described above and/or using routine laboratory procedures. The numeral  
10 designations under "Protocol" represent the following:

15

1. Tissue (suspension) was plated on filters, bombarded and then filters were transferred to culture medium. After 2-7 days, the filters were transferred to selective medium. Approximately 3 weeks after  
bombardment, tissue was picked from filters as separate callus clumps onto fresh selective medium.

20

2. As in 1. above, except after bombardment the suspension was put back into liquid - subjected to liquid selection for 7-14 days and then pipetted at a low density onto fresh selection plates.

25

3. Callus was bombarded while sitting directly on medium or on filters. Cells were transferred to selective medium 1-14 days after particle bombardment. Tissue was transferred on filters 1-3 times at 2 weeks intervals to fresh selective medium. Callus was then briefly put into liquid to disperse the tissue onto selective plates at a low density.

30

4. Callus bombardment. The tissue was transferred onto selective plates one to seven days after DNA introduction. Tissue was subcultured as small units of callus on selective plates until transformants were identified.

Table 9

Expression Cassette	Protocol	Gene in Callus	Callus Expression	Gene in Ro Plant	Ro Plant Expression	Gene in Progeny Plant	Progeny Plant Expression
<i>uidA</i> (GUS, reporter gene)	1,2,3,4	+	+	+	+	+	+
<i>bar</i> (bialaphos resistance, selectable marker)	1,2,3,4	+	+	+	+	+	+
<i>lux</i> (luciferase reporter, gene)	2	+	+	+	+	In progress	
<i>hyg</i> (hygromycin resistance, selectable marker)	4	+	+	+	+	+	+
35S- <i>adh-aroA</i> (Glyphosate tolerance)	2	+	+	+	+	+	+
$\alpha$ -tubulin- <i>aroA</i> (Glyphosate tolerance)	2	+	+	+	+	In progress	
2xhis- <i>aroA</i> (Glyphosate tolerance)	2	+	-	ND	ND	In progress	
35S- <i>his-aroA</i> (Glyphosate tolerance)	2	+	-	ND	ND	In progress	
<i>R, C1</i> (anthocyanin pigment synthesis)	1	+	+				
35S- <i>laB6</i> (Bt)	2	+	ND	+	ND	In progress	

Expression Cassette	Protocol	Gene in Callus	Callus Expression	Gene in Ro Plant	Ro Plant Expression	Gene in Progeny Plant	Progeny Plant Expression
35S-HD73 (Bt)	4	+	-	+	-	+	ND
35S-1800b (Bt)	2,4	+	-	+	+	+	+
5 2730CS-AdhVI-1800b (Bt)	2	+	ND	+	+	In progress	
35S-Adh1-1800b (Bt)	2,4	+	ND	+	In progress		
35S-MZTP-1800b (Bt)	2,4	+	In progress				
Adh1-adh1-1800b (Bt)	4	+	+	+	-	Completed	
10 potato <i>pinII</i> (proteinase inhibitor confers insect resistance)	2	+	+	+	ND	In progress	
15 tomato <i>pinII</i> (proteinase inhibitor confers insect resistance)	2	+	ND	+	ND	In progress	
35S- <i>dapA</i> (altered lysine production)	3,4	+	+	In progress			
20 Z27- <i>dapA</i> (altered lysine production in seed)	3,4	+	NA	ND	ND	In progress	
25 Z27Z10 (altered storage protein in seed)	3,4	+	NA	ND	NA	+	+
Z4Z10 (altered storage protein in seed)	3,4	+	NA	ND	NA	In progress	
25 Z10Z10 (altered storage protein in seed)	3,4	+	NA	ND	NA	In progress	



Expression Cassette	Protocol	Gene in Callus	Callus Expression	Gene in Ro Plant	Ro Plant Expression	Gene in Progeny Plant	Progeny Plant Expression
10Z4ENT (altered storage protein in seed)	3,4	+	NA	ND	NA	In progress	
10Z0P (altered storage protein in seed)	3,4	+	NA	ND	NA	In progress	
5 35S- <i>adh1-mtID</i> (enhanced stress resistance)	2	+	In progress				
<i>deh</i> (resistance to dalapon herbicide)	4	ND	+	In Progress			

10

NA indicates not applicable, e.g., gene does not express in that tissue type.

ND indicates not done, but tissue was available.

15 Blank space indicates experiment has not progressed to this point or was terminated before this point.

The symbol " + " indicates that expression of the gene was detected by RNA, protein, enzyme assay or biological assay.

## 1. Herbicide Resistance

**Example 35: Glyphosate resistance--Transformants Containing the *Salmonella typhimurium aroA* gene.**

5

This example describes certain methods relating to the use of an *aroA* gene construct in maize transformation. The herbicide glyphosate acts by inhibiting the enzyme EPSP Synthase. EPSP Synthase is presents in plants and bacteria and the gene used in this example, *aroA*, was isolated from *Salmonella typhimurium*.

10 Certain mutated versions of the *aroA* gene are known which encode variant EPSP Synthase enzymes which are insensitive to glyphosate (Comai et al., 1983).

Transformation studies were conducted employing pDPG238, a tandem *bar-aroA* construct containing a Calgene *aroA* plant expression cassette.

15 Transformation using the SC716 culture yielded four clones that produced forty-five plants in the greenhouse. Results from PCR analysis demonstrated that three of these four clones contained the *aroA* gene and one line also expressed the gene at the limit of detection by Western analysis. A plant from this clone was pollinated, two embryos rescued and two R<sub>1</sub> plants grown (designated TGB-4 and  
20 TGB-5). Both R<sub>1</sub> plants contained the *aroA* gene as determined by PCR analysis and one plant (TGB-5) expressed the gene as determined by Western analysis. Progeny of the *aroA* expressing plant (TGB-5) were included in field tests.

25 An experiment was conducted to examine the level of resistance to glyphosate (Roundup®) in crosses of the *aroA* expressing line TGB-5 produced using pDPG238. This line contains both the *bar* and *aroA* genes and hence is expected to confer resistance to both Basta® and Roundup®. A single progeny of TGB-5, designated TGB-56, was crossed to four elite inbreds, representing four different heterotic groups. The progeny were grown and self-pollinated to increase  
30 seed, and the resultant seeds were planted in two experiments. The first

experiment was sprayed with 1.5 lb/A Basta® to confirm Mendelian segregation of the introduced DNA (Table 10). The remaining plants were divided into four blocks and sprayed with application rates of Roundup® of 2 oz/A, 4 oz/A, 8 oz/A, and 16 oz/A. All plants were killed in the 8 oz and 16 oz treatments (normal field application rates for weed control). At 4 oz/A a portion of the transformed plants were killed and a portion were stunted in growth. The ratio of dead plants to slow growing plants was not significantly different from the expected 3:1 ratio in progeny from three of the four self pollinations (Table 11), indicating that there was a low level of expression of the *aroA* gene providing partial resistance to herbicide application.

TABLE 10: Segregation of Basta® resistance.

		<u>Resistant</u>	<u>Susceptible</u>	
15	(CD x TGB-56)	128	56	$\chi^2 = 1.22$
	(AW x TGB-56)	129	52	$\chi^2 = 1.10$
	(CN x TGB-56)	136	48	$\chi^2 = .075$
	(AF x TGB-56)	107	44	$\chi^2 = 1.17$

TABLE 11: Segregation of Roundup® Resistance.

		<u>Resistant</u>	<u>Susceptible</u>	
	(CD x TGB-56)	63	26	$\chi^2 = 0.739$
	(AW x TGB-56)	66	30	$\chi^2 = 1.680$
25	(CN x TGB-56)	64	25	$\chi^2 = 0.005$
	(AF x TGB-56)	53	30	$\chi^2 = 4.430$

Studies involving the E1 cell line yielded nine clones that have produced sixty-five plants in the greenhouse. Results from PCR analysis demonstrated that five of these clones contain *aroA*, and Western analysis of leaf tissue indicated that certain plants (clone #58) express *aroA*. No R<sub>1</sub> progeny were recovered from E1 plants.

Several tandem *bar-aroA* vectors were utilized in which *aroA* expression units had been introduced into pDPG295 and pDPG298, as described earlier (see section E, DNA segments). Briefly, these include vectors with a 35S-histone promoter fusion, in which the genes are placed in convergent, divergent, and colinear orientations (pDPG314, pDPG313, and pDPG317, respectively) with respect to the *bar* expression cassette; colinear and divergent vectors employing a histone promoter (pDPG315 and pDPG316, respectively); and colinear and divergent vectors employing an  $\alpha$ -tubulin promoter (pDPG318 and pDPG319, respectively). DNA of five of these constructs was prepared for bombardment (pDPG313, pDPG314, pDPG315, pDPG317, pDPG319).

It was firstly determined which of the tandem *bar-aroA* orientation-promoter combinations functioned best under the experimental employed. The 5 DNA constructs, pDPG313, pDPG314, pDPG315, pDPG317, and pDPG319, were bombard into the E1 cell line. Many transformed clones were recovered which revealed that no construct was more useful than any other using bialaphos selection. PCR analysis showed that co-transformation frequencies were about identical (75%) regardless of which construct was used. It was therefore concluded that all constructs were functioning, and that no one construct was better than another for transformation. Clones from these studies were cryopreserved for future analysis.

Since all constructs appeared to be functioning similarly, efforts were concentrated on using the 3 divergently constructed tandems pDPG315, pDPG317,

and pDPG319 in which the *aroA* gene is driven by the 2X histone, Camv 35S-histone, and  $\alpha$ -tubulin promoters, respectively. Bombardments were initiated using 16 new cell lines (other than E1). These lines include AT824, SC716, E4, ABT4, and various other new A188 x B73 and B73 x A188 cultures. *aroA* transformants were successfully recovered from cell lines AT824, SC716, and E4 (A1880 cell culture revived from EniMont cryopreservation). Regeneration was begun on *aroA* confirmed clones of all three lines. Regenerated plants from the AT824 and SC716 clones are in the greenhouse. Four SC716 transformants containing the  $\alpha$ -tubulin promoter-*aroA* expression vector produced R<sub>1</sub> seed. AT824 transformants produced R<sub>1</sub> seed as follows: five transformants containing the  $\alpha$ -tubulin promoter, two transformants containing the CaMV 35S-histone fusion promoter and six transformants containing the 2x histone promoter. These transformants are currently in field tests to determine levels of glyphosate resistance.

#### **Example 36: Tissue Specific Expression of *aroA* in Roots of Transgenic Plants**

Transformants were maintained on medium 223 (Table 1). For regeneration cells were transferred to medium 189 (Table 1) and cultured in the dark. Cultures were subcultured two weeks later onto fresh medium 189 (Table 1). Regenerating tissue was transferred to medium 101 (Table 1) in low light, followed by rooting of shoots on medium 607 (Table 1) or 501 (Table 1) and transfer to Plant Cons®. Rooted plants were grown hydroponically to avoid soil and microbial contamination when attempting to assay plants for root specific expression of the EPSPS gene.

Expression of the EPSPS gene, *aroA*, was assayed by Western blot analysis. Leaf and root samples were harvested from transgenic plants. Approximately 1 gram of tissue was ground in a glass homogenizer with 400  $\mu$ l RIPA buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, 50 mM Tris-HCl pH 8.0). Extracts were centrifuged at 14,000 x g and supernatants collected for protein analysis. Forty  $\mu$ l of each protein extract was run on a 12.5% polyacrylamide denaturing gel

(Laemmli, 1970). The gel was run overnight at 50 volts. Following running the gel was electroblotted to nitrocellulose paper and the nitrocellulose dried at 37C. The blot was washed in 50 ml 5% nonfat dry milk (NFD) in TBS (20 mM Tris-HCl pH 7.5, 0.5M NaCl) for 30-60 minutes followed by incubation overnight in 50 ml 5% NFD, TBS containing 100 ul EPSPS rabbit antiserum. The nitrocellulose blot was washed 3 times for 5 minutes each in TBS and then incubated in 50 ml 5% NFD, TBS containing 100 ul goat anti-rabbit antiserum conjugated to horseradish peroxidase for two hours. The nitrocellulose blot was washed 3 times with TBS for 5 minutes each prior to staining. The blot was stained as follows. Twenty four mg 4-chloro-1-naphthol was dissolved in 8 ml methanol. Forty two ul 3% H<sub>2</sub>O<sub>2</sub> was added. Thirty minutes after initiation of staining 400 ul of H<sub>2</sub>O<sub>2</sub> was added. The staining reaction was stopped by adding water and drying the blot. Western blots were stored in the dark.

Expression of EPSPS was detected in roots derived from plants of transformant S10AV13. No expression was observed in leaf tissue. The protein expressed in the root was identical in size to EPSPS protein isolated from *Salmonella typhimurium* and run on the same gel as a positive control. This is the expected expression profile expected, because the  $\alpha$ -tubulin promoter is root specific in maize. The *aroA* expression cassette in pDPG319 also contains a transit peptide of about 130 amino acids to target the EPSPS protein to the plastids. As the protein expressed in maize is identical in molecular weight to the protein isolated from *S. typhimurium* it is apparent that the transit peptide has been correctly cleaved from the EPSPS protein. Hence it is believed that the EPSPS protein was targeted to the plastids as demonstrated in example 7.

### Example 37: Herbicide Application (Basta®)

The herbicide formulation used, Basta TX<sup>R</sup>, contains 200 g/l glufosinate, the ammonium salt of phosphinothricin. Young leaves were painted with a 2% Basta solution (v/v) containing 0.1% (v/v) Tween-20. The prescribed application rate for this formulation is 0.5-1%.

In Figure 8A, Basta<sup>R</sup> solution was applied to a large area (about 4 x 8 cm) in the center of leaves of a nontransformed A188 X B73 plant (left) and a transgenic R<sub>0</sub> E3/E4/E6 plant (right). In Figure 8B, Basta was also applied to leaves of four R<sub>1</sub> plants; two plants without *bar* and two plants containing *bar*. The herbicide was applied to R<sub>1</sub> plants in 1 cm circles to four locations on each leaf, two on each side of the midrib. Photographs were taken six days after application.

### Example 38: Resistance in the Field to the Herbicide Ignite®/Basta®

Experiments have been undertaken to determine whether transformants containing the *bar* gene exhibit sufficient levels of herbicide resistance to be useful commercially. Eleven independent *bar* transformants were evaluated for field levels of resistance to the herbicide Basta® (a.k.a. Ignite®). The field design used a split-split plot with 2 repetitions. Whole plots were spray rates (1x, 3x and 7x) and subplots were transformant sources. Transformant sources were planted in four-row plots with two rows sprayed with Ignite and two rows sprayed with water. A spray rate of .33lb/A (1x) was used to test for efficacy. This will probably be the field rate for weed control in corn fields.

The range of responses was too narrow and field variation too large for a rating system to be useful for evaluating field levels of resistance. At the normal field application rate of Ignite® all transformant sources demonstrated resistance to

the herbicide. Differences were observed between the transformants at an application rate that was three times the normal rate. Each plot was examined by comparing sprayed versus unsprayed rows for 1) leaf-necrosis in the whorl where the herbicide accumulated at spraying; 2) chlorosis of the whorl tissue; 3) abnormal leaf growth in the whorl after spraying; 4) variability and stunting of sprayed planting compared to unsprayed; and 5) overall reduction in plant growth of sprayed versus unsprayed rows.

Differences in herbicide sensitivity were not dramatic, but consistent enough to rank each transformant , i.e. all transformant showed resistance to the herbicide. Transformants could be roughly classified into three major response groups with little difference between sources within the group . Transformants A24, B16 and E29 were most resistant to the herbicide. The sources A24 and B16 were the best, with no phenotypic difference between sprayed and unsprayed plots. Source E29 was also very good, but was somewhat variable or slightly shorter in sprayed plots. Sources A18, V11, and E19 were intermediate in response to Ignite®. The sprayed rows were slightly shorter, but were uniform and did not have any of the phenotypic abnormalities seen in the more sensitive transformants. Transformant sources G18, G20, K20, E14 and E27 were clearly sensitive to the 3x application rate of Ignite®. They were shorter and had plants with necrotic lesions, chlorotic whorls and leaf abnormalities group.

These experiments clearly demonstrate that transformants containing the *bar* gene are useful for production of herbicide resistant commercial hybrids.



## 2. Grain Quality

### Example 39: Elevation of Lysine Levels in Maize Grain

5           As described in the previous U.S. Patent Application No. 07/204,388 (most claims of which have been granted), one approach to enhancing lysine levels in maize grain involves lysine overproduction through deregulation of the lysine biosynthetic pathway. A key regulatory point in the lysine biosynthetic pathway occurs at the condensation reaction in which pyruvate and aspartyl semialdehyde  
10       form dihydrodipicolinic acid. This reaction is catalyzed by the enzyme dihydrodipicolinic acid synthase (DHDPS), which is normally feedback-inhibited by free lysine. In that previous patent application, data were presented which demonstrate that expression of a lysine-tolerant version of DHDPS, encoded by the *E. coli* *dapA* gene, in transgenic tobacco plants leads to elevated lysine levels in  
15       plant cells. As presented below, we have transferred similar gene constructs to maize cells and have successfully regenerated transgenic plants which contain these *dapA* gene constructs and express the lysine-tolerant DHDPS in maize seeds.

          Plasmid constructs were introduced, in various combinations, into maize  
20       cells by particle bombardment as described above. Transgenic cell lines were identified on the basis of resistance to the appropriate selectable agent, either hygromycin (Hyg), phosphinothricin (Ppt), or bialophos (Blp), included in the growth medium. These lines were then screened at the callus level for presence of appropriate DNA sequences by PCR amplification assays. Several cell lines have  
25       been established which are at various stages of plant regeneration. Current status of transformed plants that have been transferred to soil is summarized in the following Table 12:

Table 12

DPG plasmid constructs used for bombardment	Recipient cell line	Selection agent	Transgenic line(s) carried through plant regeneration	Status
334/367	AB61	Hyg	HAL	R2 seed
334/366	AB61	Hyg	Dap2	R2 plants
334/371/367	AB61	Hyg	Dap3	R2 plants
335/355	HAL	Ppt	Dap4	R1 seed
371/363	ABT4	Ppt	ND1-1 ND1-4	R1 plants
335/372/231	ABT4	Blp	dAH03CF-10 dAH06CF-10 dAH06CF-15 dAH09CF-11	R0 plants
335/372/231 335/372/283	ABT4	Blp	dAH04CG-11 dAH07CF-11 dAH07CF-12 dAH07CF-15 dAH07CF-19 dAH07CF-24 dAH07CG-17	R0 plants
335/165	HB13-3	Blp	dAU01CG-10	R0 plants

For each of the most recent experiments, where only R0 plants have been generated to date, several additional cell lines are in the plant regeneration process. In these experiments, in which the main objective is to obtain expression of the

DSTP/dapA transgene in various tissues, several dozen transformants have been obtained by co-bombardment with multiple plasmids. Only those lines which, when assayed at the callus level, are PCR-positive for the selectable marker transgene and the dapA construct(s) of interest are transferred to the plant regeneration program. In summary, these experiments have yielded the following numbers of bar-positive transformants:

5

<u>Genotype by PCR assay</u>	<u>No. transformants</u>
DPG 335/372	35
DPG 335	21
DPG 372	26
DPG418	20

10

15

PCR-amplification assays of DNA extracted from callus samples were performed by standard procedures. In each assay, one oligonucleotide primer (primer 1 in the table below) was specific to the promoter present in the transgene, and a second primer (primer 2) corresponded to the dapA coding region or, for pDPG418, the Glb1 3' sequence. Designations of the primers used for the PCR-amplification assays of the dapA constructs, along with the sizes of the amplified fragment products, are provided in the following Table 13:

20

**Table13**

Construct	Primer 1	Primer 2	Fragment size
334 Z10/MZTP/dapA/nos	Z10P965	dapAPCR1	572 bp
335 Z27/DSTP/dapA/35S	Z27mid	DAP6	1011bp
371 35S/MZTP/dapA/nos	35SPCR1 35SPCR1	dapAPCR1 DAP6	480 bp 610 bp
372 35S/DSTP/dapA/nos	35SPCR1 35SPCR1	dapAPCR1 DAP6	486 bp 616 bp
418 Glb1/DSTP/dapA/Glb1	Glb15' Glb15' Glb15'	Glb13' dapAPCR1 DAP6	1300 bp 464 bp 594 bp

Expression of dapA transgenes in transformed plant cells has been analyzed to date primarily by assaying for the presence of lysine-tolerant DHDPS activity essentially as described in U.S. Patent Application Serial No. 07/204,388, except that modifications have been made for use of the assay in a qualitative manner in a microtiter plate format as follows: a few milligrams of each tissue sample are placed in the wells of a 96-well plate with 50 ul of 0.225 M tris, 17.3 mM sodium pyruvate, pH8.2, covered, then transferred to a -20°C freezer for at least one hour. After the samples are thawed briefly, 50 ul of the reaction mix described by Yugari et al (J. Biol. Chem, 240:4710-4716, 1965), supplemented with L-lysine to 0.45 mM, was added and the reactions were incubated at 37°C for 1-2 hours. Reactions were quenched by the addition of 175 ul stop buffer, and pink color was allowed to develop at room temperature for 15-60 minutes, at which time the samples were scored as plus or minus lysine-tolerant DHDPS activity on the basis

of presence or absence, respectively, of pink color. This assay has been applied in this form to portions of cultured maize callus, portions of immature seeds, fragments of mature dry kernels, and leaf sections from plants at various stages of growth. Results of lysine-tolerant DHDPS expression assays in the transgenic maize lines from which mature plants have been obtained are summarized in the following Table 14:

**Table 14**

<b>Transgenic line</b>	<b><i>dapA</i> promoter construct(s)</b>	<b>Callus</b>	<b>Leaf</b>	<b>Immature kernels (20 DAP)</b>	<b>Mature kernels</b>
HAL	334 (Z10)	no	nd	nd	nd
Dap2	334 (Z10)	no	no	yes	yes
Dap3	334 (Z10) 371 (35S)	nd	no	yes	yes
Dap4	335 (Z27)	nd	nd	nd	nd
ND1-1	371 (35S)	yes	nd	nd	n.d.
ND1-4	371 (35S)	yes	yes	nd	no

The expression of the *dapA* transgene in both developing and mature maize kernels from transgenic plants is significant with respect to use of these, and related, gene constructs in development of high-lysine maize types through deregulation of the lysine biosynthetic pathway. This trait is transmitted and expressed at least through the R2 generation, as R2 seeds of both Dap2 and Dap3 contain lysine-tolerant DHDPS activity.

The following additional lines have been assayed for lysine-tolerant DHDPS activity at the callus level. These lines have either produced plants that have recently been transferred to soil or are in the early stages of plant regeneration (Table 15).

5

**Table 15**

Cell line	<i>dapA</i> construct(s)	Lys-tolerant DHDPS activity
dAH03CF-10	335/372	+
dAH06CF-10		+
dAH06CF-15		-
dAH09CF-11		-
dAH04CG-11	335/372	-
dAH07CF-11		-
dAH07CF-12		+
dAH07CF-15		-
dAH07CF-19		-
dAH07CF-24		-
dAH07CG-17		+
dAU01CG-10	335	+

10

15

Cell line	<i>dapA</i> construct(s)	Lys-tolerant DHDPS activity
dAR01E1-10	418	-
dAR01E1-11		+
dAR01E1-13		+
dAR01E1-14		+
dAR01E1-15		-
dAR01E1-16		+
dAR01E1-17		-
dAR01E1-18		+
dAR01E1-19		+
dAR01E1-20		+
dAR01E1-21		+
dAR01E1-23		-
dAR01E1-24		-

Expression of the *dapA* transgene in seeds of transformed plants as described above is very encouraging with respect to our goal of deregulating lysine biosynthesis in maize kernels. To date, expression of the lysine-tolerant *dapA* gene product has been accomplished by using the endosperm-specific promoters Z10 and Z27, and it is anticipated that use of the embryo-specific *Glb1* promoter will result in expression of the *dapA* gene product in embryos as well.

#### **Example 40: Enhanced Methionine Content of Maize Seeds**

The purpose of these experiments is to enhance methionine content of maize kernels for improved poultry feed. This goal is achieved through particle bombardment of maize cells with DNA-coated microprojectiles and subsequent

selection of transformed cells, followed by regeneration of stably transformed, fertile transgenic maize plants which transmit the introduced genes to progeny. The gene used for enhanced methionine content encodes a 10 kD zein seed storage protein which is 23% methionine, and seeds of transformed plants overexpress this gene, leading to increased 10 kD zein and increased methionine content.

The zeins are a large family of related proteins which accounts for more than 50% of the total protein in maize seeds. The  $\alpha$ -zeins, which are low in lysine, methionine and tryptophan, are the most abundant of the zeins. Thus, maize seeds are deficient in these amino acids because such a large fraction of the total protein is  $\alpha$ -zein. One method to correct for this deficiency, and to substantially increase the seed levels of various amino acids, especially methionine, is to overexpress a gene encoding a 10 kD  $\delta$ -zein containing 23% methionine.

U.S. patent application 07/636,089, filed December 28, 1990, describes the production of transgenic *Zea mays* plants and seeds, which have been transformed with recombinant DNA encoding the 10 kD  $\delta$ -zein. Transgenic plants are obtained by bombardment of friable, embryogenic callus with microprojectiles coated with recombinant DNA encoding the 10 kD zein and a selectable marker gene, followed by selection of transformed callus and regeneration of fertile plants, which transmit the introduced gene to progeny.

A transformed cell line, designated Met1, was obtained by bombarding AB63S cells with the plasmids pDPG367, pDPG338, and pBII221. Selection was on 60 mg/l hygromycin, and the presence of the HPT, GUS and Z27Z10 genes was confirmed by PCR analysis. Additionally, the presence of the HPT coding sequence and the Z27Z10 gene was confirmed by Southern analysis. Met1 exhibited strong resistance to hygromycin, and was only 20% inhibited at 200 mg/l hygromycin. Thirty two additional lines carrying methionine constructs were identified (using



selection procedures described elsewhere in this CIP) as shown in Table 16.

Table 16. Genotypes of Cell Lines PCR<sup>+</sup> for Methionine Constructs

5

	<u>Cell Line</u>	<u>Genotype</u>
	Met1	Z27Z10
10	MD 64-1	Z27Z10, Z10Z10
	MD 84-34	Z27Z10, Z4Z10
	MD 84-31	Z4Z10, Z10Z10
	MD 84-2	Z4Z10
	MD 52-8	Z4Z10, Z10Z10
15	MD 52-11	Z27Z10, Z4Z10, Z10Z10
	MD 52-10	Z4Z10
	MD 44-2	Z4Z10, Z10Z10
	MD 42-1	Z27Z10, Z4Z10, Z10Z10
	MD 32-2	Z10Z10
20	MD 32-1	Z27Z10, Z10Z10
	A6-101	Z27Z10, Z4Z10, Z10Z10
	A6-115	Z27Z10, Z4Z10, Z10Z10
	A6-181	Z27Z10, Z10Z10
	A6-151	Z27Z10, Z4Z10, Z10Z10
25	A6-161	Z4Z10, Z10Z10
	A6-907	Z27Z10, Z4Z10, Z10Z10
	A8-113	Z10Z10
	A8-301	Z27Z10, Z4Z10, Z10Z10
	A10-2	Z27Z10, Z4Z10, Z10Z10
30	A10-7	Z27Z10, Z4Z10, Z10Z10

	B1-72	Z27Z10
	B1-82	Z27Z10
	B1-94	Z27Z10
	B1-101	Z27Z10
5	B1-401	Z27Z10
	B1-601	Z27Z10
	B1-702	Z27Z10
	B1-703	Z27Z10
	B1-703	Z27Z10
10	B1-704	Z27Z10
	B1-705	Z27Z10
	B1-901	Z27Z10

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15           Transformants were identified by PCR analysis. The Z27Z10 chimeric gene was distinguished from endogenous genes by generation of a PCR product which spanned the junction of the Z27 promoter and the Z10 coding sequence. Similarly, the Z10Z10 introduced gene was identified by a PCR product which spanned the junction of the pUC plasmid which carried the construct and the Z10 promoter, and

20           the Z4Z10 construct was identified by a PCR product spanning the junction between the Z4 promoter and the Z10 coding sequence.

25           Forty five plants were regenerated from Met1 callus. These plants were selfed and reciprocal crosses were made using 6 inbred lines. Immature seed was harvested at 21-24 DAP for Northern analysis, and genotype of the progeny was also examined by PCR. In all cases, the HPT and Z27Z10 genes cosegregated, consistent with Mendelian segregation of a single locus, and indicating linkage of the introduced HPT and chimeric Z27Z10 genes. Additionally, the GUS gene was

30           shown to cosegregate with the HPT and Z27Z10 genes, allowing the use of a GUS

assay to be used to identify the Z27Z10 genotype. Northern analysis confirmed that the chimeric Z27Z10 gene was expressed only in seeds PCR<sup>+</sup> for the Z27Z10 gene. As with PCR analysis, the presence of the Z27Z10 transcript was confirmed using a probe which spanned the junction between Z27 and Z10 sequences.

5

ELISA analysis of Met1 R<sub>1</sub> seed using a Z10-specific antibody revealed a trend of increased 10 kD zein levels as compared to controls. Seeds carrying the Z27Z10 gene showed 2 to 3-fold higher levels of 10 kD zein per unit protein than nontransformed seed. Even more striking results were obtained in ELISA analysis of R<sub>2</sub> seeds. In these experiments, embryos were isolated from seeds and germinated, and PCR analysis for the Z27Z10 gene was carried out on the seedlings from the excised embryos, and protein analysis was carried out on the remaining seed tissue. The Z10 gene product accounted for up to 0.6% of the dry seed weight, or 6% of the total protein. An average 7-fold increase in 10 kD zein expression was found. Field test data from 90 bulked R<sub>3</sub> seed samples indicated a positive correlation between elevated 10 kD zein levels and the presence of the Z27Z10 construct. For ELISA analysis, zeins were extracted from corn meal samples in 70% ethanol and 2% B-mercaptoethanol at room temperature. Extracts were dried down on 96-well microtiter plates and incubated sequentially with 1% BSA, primary antibody, peroxidase-conjugated secondary antibody, and enzyme substrates (for peroxidase). Absorbances at 490 minus 410 nanometers were collected, and a standard curve using purified 10 kd-specific antibody was used to calibrate each plate. Three extractions were carried out for each sample, and each extraction was assayed in 3 wells. Thus, 9 absorbance measurements were made for each sample.

25

Protein levels were measured by near infra-red reflectance spectroscopy, and methionine levels were measured by oxidation of meal, followed by acid hydrolysis with detection of the released methionine sulfone by PITC (phenyl- isothiocarbamate) pre-column derivitization and reverse-phase HPLC. The presence

30

of Z27Z10 DNA was confirmed by PCR and/or Southern analysis.

PCR, 10 Kd zein, methionine and protein analyses were carried out on field test samples from 65 ears. Of the three lines analyzed, it was demonstrated that PCR<sup>+</sup> ears contained higher levels of 10 Kd zein, regardless of genotype. In addition, a correlation was shown between increased Z10 gene product, as determined by ELISA analysis, and elevated methionine levels in the seed, as determined by amino acid analysis.

#### 10 **Example 41: Improved Protein and Starch Content of Maize Seeds by Antisense DNA**

15 The purpose of these experiments is to improve the nutritional content of maize kernels by reducing the expression of  $\alpha$ -zeins, with a concomitant increase in the levels of other proteins or starch. The reduction in  $\alpha$ -zein expression is to be achieved by particle bombardment of maize cells with microprojectiles coated with antisense genes to the 19 and 22 kD  $\alpha$ -zein families, to reduce translation of  $\alpha$ -zein mRNA.

20 The majority of the zein proteins, which account for over 50% of the total seed protein, are the 19 and 22 kD  $\alpha$ -zeins. The high levels of  $\alpha$ -zeins, which are low in methionine, lysine and tryptophan, result in seeds low in these amino acids. Maize seed protein levels are inversely correlated with starch, thus reduced  $\alpha$ -zein levels would potentially result in increased starch. Increased levels of other  
25 proteins are also associated with reduced levels of  $\alpha$ -zeins in opaque and floury mutants, which reduce levels of  $\alpha$ -zeins.

30 The  $\alpha$ -zeins are encoded by a large multigene family with regions of sequence homology. Consequently, a small number of introduced antisense genes (with transcripts complementary to the conserved regions of homology in  $\alpha$ -zein

transcripts) would likely be needed. Introduction of antisense genes and selection for reduced  $\alpha$ -zein content and increases in starch or other proteins of interest, would be followed by introduction of these selected lines into a breeding program to optimize desirable characteristics. It is believed that, in light of the present disclosure, one of skill in the art would now be able to alter the nutritional content of maize seeds through transformation of maize plants using genes encoding antisense genes to the  $\alpha$ -zeins.

Two  $\alpha$ -zein antisense genes to the 19 kD zein (A20 gene) and 22 kD zein (Z4 gene) under control of the 10 kD zein promoter have been used to transform maize cells. These two plasmids were chosen from a variety of plasmids containing antisense sequences to  $\alpha$ -zeins based on reduction of  $\alpha$ -zein protein levels following hybrid arrest of  $\alpha$ -zein RNA using antisense transcripts, and *in vitro* translation of  $\alpha$ -zein mRNA not removed by hybridization. In these experiments, *in vitro* synthesized sense and antisense RNAs were prepared and mixed using a 4:1 ratio of antisense to sense RNA. Annealing conditions were determined by the appearance of sense::antisense hybrids on agarose gels. Although RNAs were successfully translated in both wheat germ and rabbit reticulocyte systems, the rabbit reticulocyte system was shown to be more efficient. Laser densitometry was used to quantitate the results of the *in vitro* translations. Several plasmids carrying antisense genes or parts of antisense genes were examined, and the plasmids p1020p and pZ4ENT were shown to be the most efficient in these assays.

Stable transformants were obtained by bombarding maize cells with the antisense constructs and selectable marker genes, and selection was carried out as described elsewhere, using Basta, bialaphos or hygromycin. Presumptive transformed calli were screened for antisense constructs by PCR, using primers to the Z10 promoter and the nos 3' sequence. Seven lines were identified which were PCR-positive for pZ4ENT, 15 lines were PCR-positive for p1020P, and 1 line

was positive for both constructs. These lines were regenerated and crossed to various inbred lines. Further transformation experiments using pDPG165 as the selectable marker gene, resulted in an additional 13 transformants which carried p1020P, 13 with pZ4ENT, and 42 which were PCR<sup>+</sup> for both plasmids. These transformed cell lines are now being regenerated according to standard procedures.

R<sub>1</sub> seeds were collected from regenerated plants, and 110 R<sub>1</sub> seeds from 6 antisense lines were grown in the greenhouse. Seed was uniform in appearance and synchronous germination occurred at 95%. Plants appeared normal, and selection for Basta resistance was carried out one month after planting by leaf painting with 2% Basta. Twenty four resistant plants were identified in this manner. These plants were selfed and crossed to inbred lines. Immature kernels of selfed plants were harvested at 10, 12 and 16 days after pollination and frozen in liquid nitrogen for future Northern analysis of antisense constructs.

### 3. Insect Resistance

The yield and efficiency of producing grain from maize throughout the world is affected by the action of a number of insect pests. The insect pests that currently affect the US maize crop include: the European Corn Borer (*Ostrinia nubilalis*;Hbn), Southwestern Corn Borer (*Diatraea grandiosella*), Southern Cornstalk Borer (*Diatraea crambidoides*), Lesser Cornstalk Borer (*Elasmopalpus lignosellus*), the corn rootworm (*Diabrotica spp.*), the corn earworm (*Heliothis zea*), armyworms (*Spodoptera frugiperda*; *Pseudaletia unipuncta*), cutworms (e.g. black cutworm: *Agrotis ipsilon*), wireworms, assorted grubs, Chinch Bugs (*Blissus leucopterus*), Corn Flea Beetles, Billbugs, Corn Root Aphids, Corn Leaf Aphids, Corn Planthopper. As well as directly affecting growth and yield, insect feeding can also lead to increased damage due to infection by other pathogens, e.g. when the insect serves actively as a vector for the pathogen (maize chlorotic mottle virus) or passively by opening the plant tissue to infection (stalk, root and ear rot fungi). Infection of the

ear by fungi can also lead to unacceptable levels of fungal toxins (aflatoxin) in maize grain. Furthermore, grain harvested from maize can also be damaged in storage by a variety of insects (e.g. seed corn maggot, meal moths, worms, beetles and weevils).

5

The control of insect pests to prevent damage is achieved in the US mainly by adopting good integrated pest management (IPM) procedures that include the use of certain farming (and storage) practices, the use of chemical and biological control measures and the use of maize germplasm that confers resistance or tolerance to insect pests. The use of some of these aspects of IPM are not always compatible with efficient, cost-effective farming or can detrimentally impact the environment through the use of chemical insecticides. Also, while traditional breeding has produced resistance or tolerance to some insect pests, the level of resistance to several important insect pests has either been inadequate to prevent economic levels of damage or is incompatible with maintaining high yield. To circumvent these problems and to reduce the use of chemical insecticide, it would be advantageous to introduce insect resistance genes into maize from a variety of sources.

In the examples described below we have utilized the transformation process to introduce into maize plants two genes from diverse sources that can, or have the potential to, control insect damage. We have demonstrated expression and inheritance of the genes in maize and have also demonstrated that the gene(s) can be used to confer resistance to a major insect pest of maize, the European Corn Borer.

In the first example (see example 42) , DNA coding for the endotoxin from a soil bacterium, *Bacillus thuringiensis* (Bt gene), and DNA coding for protease inhibitor II protein from tomato (*Lycopersicum esculentum*) were simultaneously transformed into maize cells and plants were regenerated to produce fertile

transgenic maize plants containing one or both of the genes. In the second example (Example 43), a Bt gene alone was introduced. In both examples a selectable marker coding for resistance to the herbicide bialaphos was also introduced and used to initially identify transformants. The close genetic linkage of the herbicide resistance and insect resistance genes may provide some utility by allowing breeders to follow the inheritance of the insect resistance genes by screening for herbicide resistance.

Potential insect resistance genes which can be introduced include the *Bacillus thuringiensis* crystal toxin genes or Bt genes (Watrud *et al.*, 1985). Preferred Bt toxin genes for use in such embodiments include the *CryIA(b)* and *CryIA(c)* genes (H.Hofte and H.R. Whiteley, 1989. Microbiol. Revs. 53: 242-255).

The poor expression of the prokaryotic Bt toxin genes in plants is a well-documented phenomenon, and the use of different promoters, fusion proteins, and leader sequences has not led to sufficient gene expression to produce resistance in several plant species (Perlak *et al.*, 1991). We have previously introduced expression vectors into maize that contain the native coding sequence for the HD73 Bt gene {a representative of the *cryIA(C)* class of Bt genes}. The gene was derived by cloning from the *B.thuringiensis* strain and modified by removing the genetic elements necessary for expression in its original host and replacing them with elements known to be capable of directing the initiation and termination of transcription of other foreign genes in maize. The expression vector produced was transformed into regenerable maize cells that were eventually regenerated into plants. The transformed maize cells and plants that were recovered that contained the Bt expression vector failed to provide resistance to ECB larvae.

To reduce this factor as an influence on Bt gene expression in maize, we have synthesized new DNA sequences for the HD73 and HD1 Bt genes that code for the same amino acid sequences as their native counterparts but which replace



codons that are rarely used in actively expressed maize genes (less than 19% of the time) with codons that are most frequently used in highly expressed maize genes. The synthetic DNA sequence coded for the active portion of the Bt genes and contained approximately the first 613 codons of the Bt genes (including the f-met initiation codon; see Figures 1 and 2 for sequences). The HD73 Bt endotoxin gene was introduced into a plant expression vector similar to that previously used for the native Bt gene and also into expression vectors with modified expression control elements designed to increase expression. Other examples of modified Bt toxin genes reported by others include the synthetic Bt *CryIA(b)* gene and *CryIA(c)* genes (Perlak *et al.*, 1991).

In the current examples, genes coding for protease inhibitors have also been introduced into maize. The use of protease inhibitors to mediate resistance to insect pests has been described before (R. Johnson *et al.*, 1989.; V.A. Hilder *et al.*, 1987) but none of these genes have previously been reported to have been introduced into maize using the transformation process. The use of a protease inhibitor II gene (PIN) from tomato or potato is envisioned to be particularly useful. Even more advantageous is the use of a PIN protein in combination with a Bt toxin protein, the combined effect of which has been discovered by the present inventors to produce synergistic insecticidal activity.

The two examples cited below illustrate the utility and benefits of using the current invention to introduce insect resistance genes into maize.

#### **Example 42: Insect Resistance in Transgenic Plants**

In this example AT824 cells were bombarded with 10 ug each pDPG165, pDPG354 and pDPG44 as described in example 10. Transformants were selected similar to example 19. The bombarded cells were removed from the microprojectile gun chamber, incubated on the wet filters in the petri dish for 16-24 hours,

scraped from the filter and placed in liquid 409 medium (10ml medium in a 125 ml conical flask). Flasks were incubated at ambient temperature (20-25°C) in an orbital shaker (New Brunswick Scientific, controlled environment, incubator shaker; 125-150 rpm). Cells were subcultured (2ml PCV into 20ml medium) every 3.5 days for one week without herbicide then subcultured in fresh 409 medium plus 1mg/L bialaphos (medium 434) every 3.5 days for 2 weeks. Cells were then dispensed onto solid 425 medium (3mg/L bialaphos) at a density of 0.1ml PCV per plate and incubated in the dark at ambient temperature. Approximately 250 plates were generated per original bombarded filter and 3-5 weeks post plating colonies of cells potentially resistant to the herbicide were identified.

Bialaphos resistant transformants were transferred to fresh solid 425 medium and subcultured twice (once every two weeks) before selected samples were taken for analysis by polymerase chain reaction (PCR) to detect the presence of the Bt and/or protease inhibitor (PIN) genes

ms. <sup>B<sup>5</sup></sup> > For PCR analysis, standard protocols were followed using the following primers:

PCR primers for PIN gene were:

PIN-1 (MD-1): 5'- GCT TAC CTA CTA ATT GTT CTT GG -3'

PIN-4: (MD-4): 5'- CAG GGT ACA TAT TTG CCT TGG G - 3'

PCR primers for Bt gene were:

BTSN64: 5'- AAC CCT GAA TGG AAG TGC - 3'

BTASN506: 5'- ACG GAC AGA TGC AGA TTG G - 3'.

Forty-two of the putative transformants (Table 17) were analyzed by PCR for the Bt gene and in most cases also for the PIN gene: A number were positive for Bt or PIN alone and a majority of PCR positive transformants were positive for both genes.

Regeneration of plants was similar to example 31. To regenerate the transformed maize cells, the bialaphos resistant maize clones were passaged (every two weeks; 24<sup>0</sup>C) on the following media at 24<sup>0</sup>C:

- 5 (i) On solid 223 medium or 425 medium and maintained for 1- 10 passages (2-3 weeks per passage).
- (ii) Passaged one to three times on 189 medium (first passage in the dark; later passages in low light; 16 hours light:8 hours dark),
- (iii) Passaged one to four times on 101 medium in higher light.
- 10 (iv) Passaged one to four times on 607 or 501 medium in Plant Con<sup>®</sup> containers in higher light.

Once shoots were observed in tissue incubated on 101 medium, the light intensity (fluorescent light: 25-250mE.M<sup>-2</sup>.S<sup>-1</sup>) was increased by slowly adjusting the distance of the plates from the light source located overhead. Transformants that developed 3 leaves and 2-3 roots were then transferred to a soilless plant growth mix. In some cases indolebutyric acid (3ml of 0.3%w/v solution) was applied to the base of rootless plants to stimulate root development.

20 Plantlets in soil were incubated in an illuminated growth chamber and conditions were slowly adjusted to adapt or condition the plantlets to the drier and more illuminated conditions of the greenhouse. After adaptation/conditioning in the growth incubator, plants (R0 generation) were transplanted individually to large (5gal) pots of soil and transferred to the greenhouse.

25

## **Genotypic Analysis.**

The genotype of several of the R0 plants was further analyzed by Southern blot to determine if the transformants were independent and had Bt DNA inserted in variable locations within the maize genome. This was achieved by: (i) cutting DNA isolated from the transformants with a restriction endonuclease (e.g. *Kpn* I) which cleaves to the 3' terminus of the Bt coding sequence and does not cleave the DNA sequence 5' to the Bt gene in the vector and (ii) carrying out a Southern blot probing the DNA with a radioactively labeled DNA probe specific to the Bt gene. The size and number of the resulting bands detected by autoradiography were indicative of the location of the nearest restriction endonuclease site located in the maize genome 5' to the inserted Bt DNA sequence. These varied in size for different independent insertion events. The results showed that, for most of the transformants analyzed, the Bt DNA inserted into different sites in the maize genome and most of the transformants were independent transformants (i.e. not clonally related to each other).

## ***Demonstration of expression of Bt endotoxin and PIN genes.***

### **Insect Bioassay (resistance to European corn borer).**

The regenerated (R0) plants were grown to early whorl stage (18"-26" extended leaf height with 5-6 leaves) and infested with neonate European corn borer larvae. A 'Davis' inoculator (BIO-SERV, Frenchtown, N.J.) was used to reproducibly introduce a fixed number (80-120) of newly hatched European corn borer larvae(dispersed with corn cob grits.) into the whorl of the plants Following inoculation, the larvae were allowed to feed on the plants for 2 weeks before they were evaluated for leaf damage. This infestation provided a simulation of an infestation with first brood (first generation) European corn borer larvae.

As shown in Table 18, high levels of resistance to ECB were seen in several transformants containing the Bt gene. Since DNA, introduced into plants by a variety of methods, can insert into a wide variety of locations within the maize genome, the level of expression of any inserted gene is variable and partly dependant on the location of insertion into the genome. The average level and pattern (developmental or temporal) of expression is also dependent on the composition of the inserted DNA. This also appears to be the case for the current example, since while several of the individual transformants showed high levels of resistance to insect feeding although a lower number containing the Bt gene did not. Depending on the composition of the introduced DNA it would usually be appropriate to evaluate a number of transformants to obtain transformants with the optimum level of expression of the introduced DNA.

#### **Demonstration of transcription of Bt and protease inhibitor genes.**

The transgenic R0 plants were further analyzed to determine whether transcription (production of gene-specific RNA) of the introduced genes could be detected. Total RNA was isolated from leaf tissue excised from the transgenic maize plants and suitable negative controls and analyzed by northern blotting procedures. The RNA was first separated according to size by electrophoresis into an agarose gel (under denaturing conditions), transferred and bound to a membrane support and then hybridized with gene specific, radio-labelled probes. The probes used were:

- |    |               |   |
|----|---------------|---|
| 25 | Bt gene:      | A fragment of DNA containing the first 1364bp of the coding sequence of the synthetic Bt gene (approximately from the <i>Nco</i> I site at the 5' terminus of the Bt gene to the <i>Bam</i> HI site 1364bp into the Bt gene). |
| 30 | Tom PIN gene: | The <i>Xba</i> I- <i>Bam</i> HI fragment of pDPG344 containing the cDNA coding sequence of the tomato protease inhibitor I gene.  |

barR gene:           The *Bam* HI - *Kpn* I fragment from pDPG165 comprising the coding sequence of the barR gene.

5     After hybridization and washing to remove non-specifically bound probes, the membranes were exposed to X-ray film and analyzed. The appearance of bands representing probe hybridizations to specific species of RNA demonstrated the transcription of the introduced genes in the transgenic maize plants. The presence of Bt RNA correlated well with the appearance of resistance to the ECB larvae. In  
10    this case since expression of the Tom PIN gene was also detected the contribution of this gene to the resistance could not be determined. Other transformants that are resistant and contain only the Bt gene, with no PIN gene present, indicate that the Bt gene can significantly increase the resistance of maize to ECB larvae (see Example 43 below).

15

***Sexual competency of R0 transgenic plants containing insect resistance genes.***

20    The S23BI3602 and S23BI3702 R0 plants, containing the introduced insect resistance genes, were grown to sexual maturity and crossed to non-transgenic inbred maize lines (e.g. inbreds "CN" and "AW") either by fertilizing the non-transgenic inbred maize plants with pollen derived from transgenic maize or fertilizing the transgenic plants with pollen from non-transgenic inbreds. The techniques used to cross and self-pollinate transgenic maize plants were described  
25    in the CIP (filed April 11, 1990) of US Patent Application Serial No. 07/467983 (filed January 22, 1990). The yield of progeny was variable and depended on the inbred parent used, but sufficient seed was recovered to demonstrate that the transgenic plants were fertile.

30

***Sexual transmission, segregation and expression of insect resistance genes in progeny.***

The harvested seed (R1 generation) was harvested, planted in soil in 5 gal pots and grown in the greenhouse. When the plants had grown so they had at least one true leaf extended, a 2%(w/v) solution of a commercial formulation of bialaphos (BASTA<sup>®</sup>, Hoechst TX100) was painted on a small circle of leaf tissue and after one week the plants were evaluated for herbicide resistance. Resistance to the herbicide was identified by the lack of a browning reaction (necrosis) in the area treated with herbicide (tissue browning = sensitivity, no browning = resistance).

Following this assay, samples were taken to determine the genotype of the segregants (by PCR assay) and the plants were infested with ECB larvae to determine insect resistance phenotype. The results (Table 19) showed that the progeny inherited the Bt and PIN genes together with the resistance to bialaphos and ECB larvae. The low insect damage rating number (high insect resistance) correlated with the presence of the Bt. No significant resistance to ECB larvae above the no-Bt controls was ever been detected in transgenic maize with only the BarR gene present. Furthermore, in a separate experiment when only the pDPG354 and pDPG165 expression cassettes were maintained in the transformed cells, R0 and R1 plants (transformant S25BJ18) the insect resistance phenotype was still inherited, suggesting that the Bt gene (without PIN gene) is capable of conferring resistance to ECB larvae.

There was no independent assortment of the introduced genes, indicating that the Bt, PIN and bialaphos resistance genes in transformant S23BI36 or S23BI37 were closely linked. Resistance was inherited independent of the inbred used. Close linkage of the herbicide resistance and insect resistance will provide an advantage for the production of commercial maize seed containing the insect

resistance gene, since the presence of the Bt gene in subsequent generations can be detected and followed by screening for herbicide resistance. This will allow for screening for the Bt gene to take place in locations and times when infestation with insects is impossible or difficult (e.g. in winter nurseries).



**Table 17.**

***PCR Analysis of transgenic maize cells (S23BI31 clones) containing Bt and/or Tom PIN genes.***

5	Clone	Bt PCR	PIN PCR	Clone	Bt PCR	PIN PCR
	01	--	+	25	ND	ND
	02	--	ND	26	--	ND
10	03	--	ND	27	ND	ND
	04	--	ND	28	--	--
	05	+	+	29	+	+
	06	--	ND	30	+	+
	07	+	+	31	+	+
15	08	--	+	32	+	+
	09	SG	SG	33	+	+
	10	+	--	34	--	--
	11	+	+	35	+	--
	12	+	+	36	+	+
20	13	+	+	37	+	+
	14	+	+	38	+	+
	15	SG	SG	39	--	+
	16	+	--	40	--	+
	17	SG	SG	41	--	+
25	18	--	ND	42	+	+
	19	--	ND	43	+	+
	20	--	ND	44	+	+
	21	SG	SG	45	+	+
	22	+	+	46	--	--
30	23	+	+	47	--	--
	24	+	+	48	--	--

**Key:**

+

= target DNA present

-- = target DNA not present  
ND = not determined  
SG = tissue stopped growing.

Table 18.

**Expression of Bt gene in transgenic maize plants.**

	<b>R0 plant</b>	<b>Bt RNA</b>	<b>Resistance Rating</b>	<b>PIN RNA</b>	<b>bar RNA</b>
5	S23BI3015	+	1	+	+
	S23BI3016	+	1	+	+
	S23BI3119	+	1	+	+
10	S23BI3128	+	1	+	+
	S23BI3203	--	5	--	+
	S23BI3202	--	8	--	+
	S23BI3204	--	8	--	+
	S23BI3704	+	ND	+	+
15	S23BI3708	+	2	++	+++
	S23BI3709	+	1	++	+++
	S23BI3712	+	1	++	+++
	S23BI3715	+	1	++	+++
	S23BI3716	+	2	++	+
20	S23BI3720	+	3	ND	ND
	No-Bt control	--	5-9	--	--

**Key:**

-- = no RNA detected  
 + = low level Bt RNA  
 ++ = intermediate level of Bt RNA  
 +++ = higher level of Bt RNA  
 ND = not determined

**Resistance ratings:**

1 = highly resistant  
 9 = highly susceptible

**Table 19.** Inheritance and expression of insect resistance in progeny from  
Example 42.

Transformant {R:S RATIO}	Plant Number	Bt PCR	PIN PCR	BAR resistance	ECB resistance
S23BI3602(CN)	02	--	--	S	6
{4R:6S}	05	--		S	6
	07	ND	ND	S	ND
	08	+	+	R	1
	09	--	--	S	6
	10	+	+	R	1
	11	ND	ND	S	9
	13	--	--	S	3
	14	+	+	R	1
	15	+	+	R	1
S23BI3604(AW)	01	--	--	S	5
{6R:9S}	02	+	+	R	1
	03	ND	ND	S	9
	04	--	--	S	7
	05	ND	ND	S	ND
	06	--	ND	S	6
	07	+	+	R	1
	08	+	+	R	1
	09	+	+	R	1

Transformant {R:S RATIO}	Plant Number	Bt PCR	PIN PCR	BAR resistance	ECB resistance
	10	--	--	S	7
	11	+	+	R	1
	12	ND	ND	S	9
	13	ND	ND	S	9
	14	ND	ND	S	ND
	15	+	+	R	1
S23BI3702(bk)	01	ND	ND	S	9
{6R:5S}	02	+	ND	R	1
	03	+	+	R	1
	05	+	+	R	1
	06	+	+	R	1
	07	+	+	R	1
	08	ND	ND	S	6
	09	ND	ND	S	9
	10	ND	ND	S	9
	12	+	ND	R	1
	13	--	--	S	6
	15	ND	ND	S	9
S25BJ1801(AW)	05	+	ND	R	1
{5R:2S}	08	+	ND	R	1
	10	+	ND	R	1

<b>Transformant {R:S RATIO}</b>	<b>Plant Number</b>	<b>Bt PCR</b>	<b>PIN PCR</b>	<b>BAR resistance</b>	<b>ECB resistance</b>
	11	ND	ND	S	9
	13	ND	ND	R	3
	14	--	ND	R	1
	15	ND	ND	S	9

**Key for Table 19:**

+ = DNA present

-- = DNA not present

S = susceptible to bialaphos

R = resistant to bialaphos

ND = Not determined

ECB resistance:

1 = Highly resistant

9 = highly susceptible

### **Example 43: Insect Resistant Transgenic Plants**

Microprojectiles were coated with DNA as described in Example 10 except 14ul of pDPG165 and 14ul of pDPG337 DNA were used. The bombarded cells were transferred (on the filter) onto solid 409 medium and moistened with 0.5ml of liquid 409 medium. Tissue was returned to liquid 401 plus coconut water one day after bombardment. Selection in liquid 409 plus 1mg/L bialaphos (434 medium) began 8 days post-bombardment and the cells were then treated as described in Example 42.

#### ***Genotype analysis.***

The data obtained using the Bt PCR primers and techniques described in Example 42 above showed that 5 out of 7 clones tested contained the Bt gene.

#### ***Regeneration of transformants.***

Clones positive for Bt were subcultured on 425 medium for 2-5 months and depending on the clone either:

- (i) passaged on 409 solid medium (1st passage about 2 months and second passage about 2 weeks) or passaged on solid 223 medium for about 19days before:
- (ii) two passages on 189 solid medium (1st for about 14-19 days and second for about 10-14 days) followed by:
- (iii) three passages on 101 solid media and;

- (iv) one to two passages on 501 solid medium (about 2 weeks per passage) in Plant Con<sup>®</sup> containers.

5      **Bioassay of R0 plants.**

Clones developing shoots and roots on 501 medium in Plant Con<sup>®</sup> containers were transferred to a soilless mix, grown in the growth room, transferred to soil and the greenhouse and assayed for insect resistance as described above. Two individual transformants, S18BF1102 and S18BF1401, were assayed for resistance to first brood ECB larvae and were given ratings of 8 and 1, respectively. Since the average rating for the no-Bt controls was about 7, S18BF1401 (one R0 plant from each clone) was considered highly resistant and S18BF1102 was considered susceptible. Since the high resistance of clone S18BF1401 to ECB larvae has not been seen for any regenerated maize plants unless they contained a Bt gene (with or without a PIN gene), we concluded that the resistance was due to the expression of the introduced Bt gene.

20      ***Sexual transmission, segregation and expression of Bt gene in progeny.***

The S18BF1401 plant was sexually crossed with inbred line "AW" and the harvested seed was germinated, grown and assayed for inheritance of bialaphos resistance phenotype, Bt genotype and ECB resistance phenotype.

Seed harvested from R0 plant (R1 seed) was planted in soil in 5 gal pots, grown in the greenhouse and assayed for resistance to bialaphos and ECB larvae as described above. Samples were taken to determine the genotype of the segregants (by PCR assay). The results (Table 20) show that the progeny inherited the bialaphos resistance and Bt genes and also



inherited the resistance to ECB larvae. The low insect damage rating number (high insect resistance) correlated with the presence of the Bt. There was no independent assortment of the selected (Bt) genes, indicating that the Bt and bialaphos resistance genes in transformant S18BF1401 were closely linked.

\*\*\*

These results show that the transformation process can be used to introduce genes that confer resistance to insects and that the genes can be inherited.

***Examples of other expression vectors.***

The structural gene or the genetic elements associated with the introduced DNA are not limited to those described in the specific examples mentioned above. We have introduced the pDPG354 vector with a vector that carries the potato protease inhibitor I gene, as well as the bialaphos resistance gene. We have also obtained transformants that contain one or more of the following Bt expression cassettes:

- (1) cassette which contains the promoter from the Adh I gene of maize, the intron I from the Adh I gene, the HD73 synthetic Bt (figure 12) gene followed by a 3' sequence containing the poly A site from the nopaline synthase (nos) gene of *Agrobacterium tumefaciens*.
- (2) cassette which contains the 35S promoter from CaMV, intron I from Adh I, synthetic Bt gene (figure 12) followed by the nos poly A.
- (3) cassette which contains the 35S promoter, Adh intron I, transit peptide derived from the maize rbcS (RuBISCO) gene fused directly to the HD73 synthetic Bt gene (Figure 12), followed by the nos poly A

sequence.

- (4) cassette which contains the 35S promoter, maize RuBISCO transit peptide fused to the synthetic Bt gene (Figure 12) such that the codons coding for the first 8 amino acids the Bt protein are substituted with the first 9 amino acids of the mature maize RuBISCO protein, followed by the 'transcript 7' 3' sequence (see Example 43).

In each case the transformants also contained the *bar* gene.

**Table 20.**

***Inheritance and expression of Bt gene in progeny of Example 43.***

	Transgenic Plant	BarR	Bt DNA	Bt RNA	ECB Resistance
5	03	R	+	+	3
	05	R	+	+	3
	07	R	ND	+	3
10	10	R	+	+	3
	13	R	+	+	3
	15	R	+	+	3
	17	R	+	ND	3
15	01	S	--	--	9
	02	S	--	--	9
	04	S	--	ND	ND
	06	S	--	--	9
	08	S	--	--	9
	09	S	--	--	6
20	11	S	--	ND	ND
	12	S	--	ND	ND
	14	S	--	ND	9
	16	S	--	ND	8
25	<b>AT824 x AW (non-transformant controls)</b>				
	1	S	ND	ND	8
	2	S	ND	ND	9
	3	S	ND	ND	9
30	4	S	ND	ND	9
	5	S	ND	ND	9
	6	S	ND	ND	9

35 **Key:** R = Resistant to BASTA; S = Sensitive to bialaphos;  
 ND = Not determined; + = detected; -- = not detected

\*\*\*

Further genes encoding proteins characterized as having potential insecticidal activity may also be used as transgenes in accordance herewith. Such genes, which could be used alone or in combinations include, for example:

1. ***Other Bt genes.***

Endotoxin genes from other species of *Bacillus thuringiensis* that are toxic either affecting viability, growth or development of the pest insects (Hofte, H. and Whitely, H.R., 1989).

2. ***Digestion inhibitors.***

Genes that code for inhibitors of the insect pest's digestive system. The gene products may themselves inhibit digestion or could code for enzymes or co-factors that facilitate the production of inhibitors: e.g. protease inhibitors such as the cowpea trypsin inhibitor (CpTI; V.A. Hilder et al.1987.) and oryzacystatin from rice (K.Abe et al., 1987 ) and amylase inhibitors such as amylase inhibitor from wheat and barley (J.Mundy and J.C. Rogers 1986 ). (For a reviews see C.A. Ryan. 1981. and C.A. Ryan, 1989).

3. ***Lectins.***

Genes that control the production of lectins (T.H.Czapla and B.A. Lang. 1990; Chrispiels et al., 1991) such as wheat germ agglutinin (Raikhol, N.V. and Wilkins, T.A., 1987) that can affect the viability and/or growth of the insect pest.

#### 4. ***Biological peptides.***

Genes controlling the production of large or small polypeptides active against insects when introduced into the insect pests, such as (i) lytic peptides (Westerhoff et al., 1989), (ii) peptide hormones (Kattaoka, H. et al., 1989; Keeley, L.L. and Hayes, T.K., 1987) and (iii) toxins and venoms (Zlotkin, E., 1985; Dee, A. et al., 1990). Such polypeptides could be synthesized in the plant as mono- or oligomers, as fusion proteins fused to carrier proteins, such as Bt, or delivered to the insects in the presence of other agents, e.g. lectins, that may stimulate uptake or activity. Biological peptides may include peptides designed by molecular modeling to interact with components in or on insects to affect growth, development, viability and/or behavior.

#### 5. ***Lipoxygenases.***

These naturally occurring plant enzymes have been shown to have anti-nutritional effects on insects and to reduce the nutritional quality of their diet. Plants with enhanced lipoxygenase activity may be resistant to insect feeding (S.S. Duffey and G.W. Felton ,1991).

#### 6. ***Production of inadequate nutrients or removal of essential nutrients.***

Genes that code for enzymes that facilitate the production of compounds that reduce the nutritional quality of the host plant to insect pests. Examples include: (i) genes that alter the sterol composition of plants may have a negative effect on insect growth and/or development and hence provide the plant with insecticidal activity. Essential sterols could be converted to undesirable sterols or

undesirable sterols could be produced directly; (ii) increasing levels or the characteristics of polyphenol oxidases and/or its substrate thereby increasing the production of toxic products or conversion of protein nutrients to undesirable by-products (Duffey, S.S. and Felton, G.W., 1991).

**7. Qualitative or quantitative changes in plant secondary metabolites.**

**DIMBOA:** It has been demonstrated that some lines of maize show resistance to first brood European Corn borer larvae due to the production of DIMBOA (Klun, J.A. et al., 1967) in the plant. There are also suggestions that DIMBOA production could have an impact on rootworm damage. The introduction of DNA that changes quantity, timing or/and location of DIMBOA production may lead to improved resistance to several maize insect pests. Candidate genes for consideration would include those involved in the pathway for production of DIMBOA, e.g. genes at the bx locus (Dunn, G.M. et al., 1981)

**Maysin:** Since maysin has been implicated in the resistance of maize to earworm (Guildner, R.C., et al., 1991) the introduction of genes that can regulate the production of maysin may be beneficial to the production of insect resistant maize. Since the current invention allows for the transfer of genes from diverse sources into maize, the invention could also make possible the transfer of the capability to produce any other secondary metabolite from any biological source into maize provided the genes needed to control the production of the metabolite are available. **Dhurrin:** genes involved in the production of dhurrin in sorghum (Branson, T.F., et al., 1969) which could be

transferred to maize and facilitate resistance to corn rootworms.

#### **8. *Genes transferred from native grasses.***

5           There are a number of native grasses that are resistant to some  
of the insect pests of commercial inbred maize are susceptible to.  
Selected landraces of *Tripsacum dactyloides* have been reported to be  
resistant to corn rootworms (Branson, T.F., 1971). Genes coding for  
the resistance trait in this species could be isolated and transformed  
10       into maize to produce rootworm resistant maize.

#### **9. *Cuticle degrading enzymes.***

15           Genes that code for enzymes that affect the integrity of the  
insect cuticle such as chitinase, protease and lipase (M.S.Goettel et  
al., 1989; J.D.G.Jones et al., 1988) could be introduced into maize to  
produce insect resistant plants. Including with these genes would be  
genes that code for activities that affect insect molting such those  
affecting the production of ecdysteroid UDP-glucosyl transferase  
20       (D.R.O'Reilly and L.K. Miller, 1989).

#### **10 *Antibiotics.***

25           Genes that control the production of antibiotics that affect  
insect viability, growth or development, e.g. genes for the production  
of nikkomycin - a compound that inhibits chitin synthesis (U.Schluter  
and G.Serfert, 1989) and avermectin and abamectin (Campbell, W.C.,  
Ed., 1989; Ikeda et al., 1987) and insecticides from fungi (P.F.Dowd  
and O.K.Miller, 1990).

### **11. Antibodies.**

Genes that can control the production of antibodies that can inhibit insects. Antibodies have been produced in transgenic plants (Hiatt, A. et al., 1989). The genes coding for the antibodies to insect targets could be cloned, engineered and transferred to maize using the current invention.

### **12. Pro-insecticides.**

Another approach that can use genes introduced into maize is the introduction of genes that code for enzymes that can convert a non-toxic insecticide (pro-insecticide) applied to the outside of the plant into an insecticide inside the plant. The benefits would be to: (i) reduce the levels of toxic insecticides applied to crops and (ii) make the insecticide very selective since it would only be converted to the insecticide inside the transgenic plants. Plants could further be engineered to degrade residual insecticide in plants parts destined for consumption by introducing genes coding for degradative enzymes that are expressed temporally or spatially to degrade the insecticide in selected tissues.

### **13. Others.**

There are numerous other genes that have the potential to facilitate resistance to insects if introduced and expressed in maize. These include: ribosome inactivating proteins (A.M.R. Gatehouse et al., 1990) genes controlling expression of juvenile hormones (Hammock et al, 1990), genes that regulate plant structures (e.g. thickness of leaves and stalks, presence of trichomes, size of root



system), the production of chemicals that can deter insect pests, act as feeding deterrents or reduce the immunity of the insect pest to disease (D.W.Stanley-Samuelson et al.,1991).

5                    In the above examples, depending on the source of the DNA, the DNA to be introduced and expressed in maize may need to be modified to obtain optimum expression (level, timing and location of expression) by changing the genetic elements that regulate expression (promoters, introns, etc.) and the coding sequence (to improve  
10 translation, RNA stability and/or gene product activity).

                  The main element required for expression of an introduced gene is the structural gene for the protein that mediates the resistance either directly as in the case of the insecticidal Bt protein, or indirectly  
15 such as in the case of an enzyme that might degrade nutrients essential for insect growth. In practice, most expression vectors will contain:(i) a promoter, located 5' to the coding sequence, to initiate transcription of the introduced gene; (ii) the DNA sequence of the gene coding for the insect resistance factor and (iii) a sequence  
20 located 3' to the coding sequence to stimulate termination of transcription. Additional sequences (enhancers, introns, leader sequences, transit or signal sequences and 3' elements (transcription terminators or poly-adenylation sites) may be used to increase expression or accumulation of the gene product.

#### 25                    **Promoters:**

                  Promoters that could be used include promoters from:

- (a)    the maize Adh I (Walker et al., 1987), cab (T.Sullivan et  
30 al.,1989. ) rbcs (Lebrun et al.,1987), PEPCase (R.L.Hudspeth

and J.W.Grula. 1989) genes;

- (b) genes that express in pollen (e.g., Hansen et al., 1989)
- (c) genes isolated from tissue-specific libraries;
- (d) any gene that is functional in maize;
- (e) pathogens that replicate in plants, especially;  
monocotyledonous plants;
- (f) synthetic promoters that utilize elements from various plant  
genes or gene from pathogens that replicate in plants;
- (g) genes that are expressed in leaves, stalks, earshanks, collar  
sheaths or roots;
- (h) genes that are expressed in leaves, stalks, earshanks. collar  
sheaths or roots but are not expressed in developing kernels.

In addition to promoters that produce adequate levels of the insect resistance gene product in the tissues eaten by the insect pest, it may sometimes be required to construct vectors that express the anti-sense mRNA of an insect resistance gene in the kernel, or other parts of the plant, in order to inhibit accumulation of the gene product in locations where it may be undesirable.

### ***Enhancers***

Transcription enhancers or duplications of enhancers could be used to increase expression. These enhancers are often found 5' to the start of transcription in a promoter that functions in eukaryotic cells, but can often be inserted in the forward or reverse orientation 5' or 3' to the coding sequence. Examples of enhancers include elements from the CamV 35S promoter and octopine synthase genes (Ellis et al., 1987). and even promoters from non-plant enkaoyotes (e.g. yeast; J. MA et al., 1988 Nature 334:631-633).

### ***Leader sequences.***

The DNA sequence between the transcription initiation site and the start of the coding sequence is termed the untranslated leader sequence. The leader sequence can influence gene expression and compilations of leader sequences have been made to predict optimum or sub-optimum sequences and generate "consensus" and preferred leader sequences (C.P.Joshi, 1987). The sequences may increase or maintain mRNA stability and prevent inappropriate initiation of translation. Sequences that are derived from genes that are highly expressed in plants, and in maize in particular, would be preferred. The leader sequence from the soybean rbcS (RuBISCO) gene was used in pDPG337.

### ***Transit or signal peptides.***

Sequences that are joined to the coding sequence of the resistance gene, which are removed post-translationally from the initial translation product and which facilitate the transport of the protein into or through intracellular or extracellular membranes are termed transit (usually into vacuoles, vesicles, plastids and other intracellular organelles) and signal sequences (usually to the outside of the cellular membrane). By facilitating the transport of the protein into compartments inside and outside the cell these sequences may increase the accumulation of gene product protecting them from proteolytic degradation. These sequences also allow for additional mRNA sequences from highly expressed genes to be attached to the coding sequence of the insect resistance genes. Since mRNA being translated by ribosomes is more stable than naked mRNA, the presence of translatable mRNA in front of the gene may increase the

overall stability of the mRNA transcript from the insect resistance gene and thereby increase synthesis of the gene product. Since transit and signal sequences are usually post-translationally removed from the initial translation product, the use of these sequences allows for the addition of extra translated sequences that may not appear on the final polypeptide.

In two examples (see section entitled "Examples of other expression vectors." above) listed above, the transit peptide sequence for the maize RUBISCO gene was fused to the Bt gene and transformed into regenerable maize cells.

### ***3' Elements.***

The most commonly used 3' elements include a sequence of DNA that acts as a signal to terminate transcription and allow for the poly-adenylation of the 3' end of the mRNA coding for the gene product. These sequences can be obtained from a number of genes that are transcribed in maize and often can be isolated from genes that expressed in other plants or pathogens that infect plants. The most commonly used 3' elements are the 3' elements from: (i) the nopaline synthase gene from *Agrobacterium tumefaciens* (M.Bevan et al., 1983. Nucleic Acids Res. 11:369-385), (ii) the terminator for the T7 transcript from the octopine synthase gene of *Agrobacterium tumefaciens* (and the 3' end of the protease inhibitor I or II genes from potato or tomato.

***Conclusions:***

5       The examples described above show that the invention can be  
used to introduce insect resistance genes into maize and that  
functional genes can be successfully inherited. Thus the invention  
shows great utility in producing transgenic maize plants of significant  
commercial value and benefit, allowing for improved resistance to  
10       insect pests, improved productivity to the farmer and improved quality  
of the environment by reducing the dependency on chemical  
insecticides.

\*       \*       \*

15       While the invention is susceptible to various modifications and  
alternative forms, specific embodiments thereof have been shown by  
way of example in the drawings and herein be described in detail. It  
should be understood, however, that it is not intended to limit the  
20       invention to the particular forms disclosed, but on the contrary, the  
intention is to cover all modifications, equivalents, and alternatives  
falling within the spirit and scope of the invention as defined by the  
appended claims.

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